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FURTHER EVIDENCE OF THE MODE OF ACTION OF VITAMIN D

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(Received for publication April 22, 1940)

Although it is a long since established fact that vitamin D influences the retention of calcium and phosphorus in the animal body, very little is known about the means by which this is accomplished. The theory of Harris and his co-workers ('31 and '32) that the chief function of vitamin D is to increase the net absorption of calcium and/or phosphorus from the gut has found some support in the more recent work of Nicolaysen ('37) and Dols et al. ('37, '38). These later workers however believe the effect on phosphorus absorption is an indirect consequence of primary interference with the absorption of calcium.

On the other hand, the belief that vitamin D functions in the intermediary metabolism of the bone-forming elements (Brown and Shohl, '30; Light, Miller and Frey, '31; Schneider and Steenbock, '39) has been supported by recent work in which an artificially produced radioactive isotope of phosphorus was used (Cohn and Greenberg, '39; Morgareidge and Manley, '39). The conclusion was reached that vitamin D had no influence on the "entrance of phosphorus into the blood but that the healing of the metaphyses of rachitic bones occurs concomitantly with a significant increase in the content of radioactive phosphorus." It is pointed out that this finding is in "contradistinction to any possible role that vitamin D may play in intestinal absorption."

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In experiments recently reported from this laboratory, ('40) it was shown that mineral oil ingestion interfered with the calcifying action of vitamin D when this vitamin was fed as cod liver oil to young dogs and to rachitic rats. The conclusion was reached that vitamin D was dissolved in and excreted with the non-absorbable mineral oil, thus preventing it from playing its part in the calcification process, whatever that role may be.

The experiments reported herein, which were planned to test further the interference of mineral oil, offer additional evidence of the mode of action of vitamin D. In these experiments, vitamin D was supplied by external irradiation of the rat, not by cod liver oil per os as in the previous study.

EXPERIMENTAL

In the first series of experiments, young rats weighing from 50 to 65 gm. were placed on Steenbock's rickets-producing ration no. 2965. When microscopic examination of the tibia of a representative rat from each litter revealed rachitic lesions (twenty-first day) the remaining rats were divided into two groups matched as carefully as possible as to litter, sex and weight, and were treated similarly except that one group was continued on diet 2965 alone and the other was given the same ration in which 10% mineral oil had been incorporated. Paired rats from each group were then exposed at the same time, in a cage especially constructed for this purpose, to the ultra-violet irradiation of a mercury vapor quartz lamp. Except during the period of irradiation which was timed to the second by means of a stop watch, all of the rats were kept in the dark and protected from natural light.

Daily exposures to the ultra-violet light were made for 5 days and on the sixth day the rats were killed and the degree of induced healing of the rachitic lesions as shown by the "line test" (McCollum et al., '22) recorded and graded according to the system of Bills and associates, '31).

In a second series of experiments, rats weighing from 50 to 65 gm. were placed immediately upon diet 2965 with and

without incorporated mineral oil and daily irradiation of both groups begun at once and continued for 18 days. They were then killed and tested for rickets by the usual method. The rickets-preventive effect of irradiation of the mineral oil-fed and non-mineral oil-fed rats was then compared by noting the degree of rickets induced in the two groups of rats. Data on the curative type of experiment are presented in table 1 and results of the preventive experiment are given in table 2.

TABLE 1

Summarized "line test" findings showing the effect of mineral oil ingestion upon the calcification of rachitic lesions induced by daily exposure to ultra-violet irradiation [1]

IRRADIATION PERIOD	DIET 2965 ALONE		DIET 2965 + 10% MINERAL OIL	
	Number of rats	Average degree of healing [2]	Number of rats	Average degree of healing [2]
seconds				
0	8	0.0	10	0.0
15	3	0.0	3	0.0
30	12	1.2	5	0.0
45	10	2.0	—	—
60	15	2.6	12	0.0
90	—	—	8	0.0
120	6	3.8	14	0.7
150			7	1.0
180			8	2.1
240			2	2.5

[1] Exposed to mercury vapor quartz lamp at a distance of 2 feet for 5 days.
[2] Graded according to the method of Bills et al. ('31).

It is quite apparent from results of both procedures that mineral oil ingestion interfered with the action of vitamin D formed by external irradiation of the rat body. "Line test" findings on the bones showed without exception that healing was absent or less advanced in the mineral oil fed group. Whereas a daily irradiation of 45 seconds was sufficient to induce 2+ healing in rachitic non-oil-fed rats, no healing of the rachitic lesions whatsoever resulted from irradiating their mineral oil-fed litter mates simultaneously and for the same length of time. Only when the exposure period was extended to 180 seconds did healing of the same order result.

On the other hand, the preventive tests showed that rickets was more advanced in the mineral oil group than in their litter mates irradiated from the beginning for the same length of time and under the same conditions. Although the development of rachitic lesions was entirely prevented by a 4-second daily irradiation of the non-oil group, the same length of exposure of the oil-fed rats resulted in severe rickets. Daily exposure for 15 seconds was required to form normal bones in the rats ingesting mineral oil.

TABLE 2

Summarized findings showing the effect of mineral oil ingestion upon the prevention of rickets by daily exposure to ultra-violet irradiation

IRRADIATION PERIOD	DIET 2965 ALONE		DIET 2965—10% MINERAL OIL	
	Number of rats	Degree of rickets after 18 days	Number of rats	Degree of rickets after 18 days
seconds				
0	5	Severe [1]	5	Severe
1	2	Moderate [2]	2	Severe
2	4	Mild or incipient [3]	4	Severe
3	4	Mild or incipient [3]	5	Severe
4	4	Normal bone	5	Severe
5	5	Normal bone	5	Moderate
7	5	Normal bone	5	Moderate
10	2	Normal bone	5	Mild or incipient
15	2	Normal bone	5	Normal bone

[1] Characterized by wide, uncalcified metaphysis.
[2] Characterized by narrower metaphysis.
[3] Characterized by swollen epiphyseal diaphyseal cartilage, but without definite metaphysis.

Thus, the presence of mineral oil in the gut seems to interfere with function of vitamin D whether that vitamin be given as cod liver oil by mouth or synthesized by ultra-violet irradiation of certain sterols in the skin. As mineral oil is probably not absorbed from the intestinal canal and therefore must cause its interference there, these results appear to indicate that the vitamin D formed by irradiation reaches the intestinal canal where at least a part of its role is to increase the absorption of calcium or phosphorus or both.

CONCLUSIONS

Mineral oil ingestion has been shown to interfere with both the healing and prevention of rickets in rats on a high calcium-low phosphorus rickets-producing ration which normally results from ultra-violet irradiation. These findings give further evidence that one role of vitamin D is to increase intestinal absorption of calcium or phosphorus either directly or indirectly.

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PIGEON ANEMIA AS A DEFICIENCY DISEASE

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(Received for publication April 25, 1940)

FIVE TEXT FIGURES AND ONE PLATE (TWO FIGURES)

The pigeon has been used extensively in studies of vitamin B (thiamin) but its requirements for other vitamins have been investigated in only a few laboratories. Williams and Waterman ('28) reported that maintenance of weight by pigeons depends on a previously unrecognized vitamin, designated as vitamin B_3 by Williams and Eddy ('28). These same authors (Williams and Eddy, '31) later expressed lack of confidence in the vitamin B_3 hypothesis, but O'Brien ('34), and Waterman and Ammerman ('35) have provided strong evidence in its support. Carter, Kinnersly, and Peters ('30) reported that the pigeon requires still another unrecognized water-soluble vitamin, which they designated as vitamin B_5.

The original purpose of this investigation was to compare the response of pigeons and rats when they receive the same simplified ration, with the idea of concentrating first on the weight maintenance factors. It soon became apparent though (Hogan, Richardson and Johnson, '37), that many of the experimental pigeons were anemic. Doan, Cunningham and Sabin ('25) reported that fasting for 10 to 18 days produced a weight loss of about 125 gm. and a lowering of the erythrocyte count from 4 to 2.75 million per cubic millimeter. Barlow ('27) observed anemia in pigeons which had been maintained on a diet of polished rice, and expressed the opinion that

this was the result of partial inanition. Barlow and Whitehead ('28) studied pigeon blood in some detail and reported that after protracted fasting, or after the development of rice disease, the erythrocytes ranged between 1 and 3.44 million per cubic millimeter.

The chief object of this paper is (1) to describe the technique used to produce the anemia and (2) to present evidence on the nature of the deficiency.

EXPERIMENTAL

Wild pigeons were trapped and used as experimental animals. They were quartered in groups of about six, in wire cages 2 feet square and 14 inches high, with screen floors of 1 inch mesh. Water and food were available at all times but the amounts consumed were not recorded. After the pigeons reached a constant weight, referred to hereafter as normal weight, they were transferred to the experimental ration, no. 1669, composed of casein 20, sucrose 71, salt mixture (Osborne and Mendel, '19) 4, cellulose 3, and cod liver oil 2% respectively.

As was mentioned in the introduction, our original intention was to study the weight maintenance factors. After these studies had been under way some 10 or 12 weeks though, it was observed that some of the pigeons had become pale about the iris. Red cell counts showed that the number of erythrocytes was much lower in all these birds than in those which received the stock ration, and an attempt was made to determine whether the condition is reproducible.

During these preliminary observations, and as is still our custom, the pigeons were first depleted of vitamin B_1 by supplying them with ration 1669 alone. On the average the pigeons lose about 30% of their initial weights by the end of the fourth week, and about 25% develop polyneuritis. Since many will die without developing typical polyneuritis, vitamin B_1, or a combination of supplements, was supplied when the weight had dropped to 70% of the normal level, even if the typical symptoms of a vitamin B_1 deficiency did not appear.

The weekly intervals indicated in the table, graphs, and text are counted from the date the pigeons were first given the experimental ration, no. 1669.

The development of anemia. The anemic condition was first observed in pigeons which were receiving a water extract of yeast that had been irradiated by the method of Richardson and Hogan ('36). At this time none of the water-soluble vitamins required by the pigeon was available in crystalline form, so our early studies were carried out with a variety of more or less crude preparations. Later, when crystalline thiamin and riboflavin became available the observations were repeated, with essentially the same result.

In the exploratory work now to be described three different vitamin B_1 carriers were used: (1) the irradiated water extract of yeast; (2) Jansen's acid clay adsorbate [1] and (3) a vitamin B_1 concentrate.[2]

The irradiated water extract of yeast alone was supplied in daily doses of 300 mg. or more of dry matter. This quantity heals polyneuritis promptly and the birds almost invariably gain in weight although they never regain normal weight unless supplied with a more complete vitamin supplement. This partial recovery may last from 1 to 5 weeks and is usually followed by a decline. The red blood cell counts may begin to fall within 3 or 4 weeks, but occasionally the number remains normal for 8 or 10 weeks. Of the twenty pigeons that received this supplement thirteen became severely anemic in periods varying from 5 to 16 weeks from the time they were placed on ration 1669. Six became mildly anemic, with blood counts ranging from 2.10 to 2.74 million per cubic millimeter, in periods ranging from 7 to 19 weeks. The other pigeon had a subnormal red cell count, from 2.80 to 3.94 per cubic millimeter, but it died after 13 weeks. It was also observed that as the anemia developed some of the erythrocytes became abnormal in shape. These abnormal cells were counted

[1] Kindly supplied by Dr. Jansen.

[2] Kindly supplied by Merck and Co.

over a considerable period of time, and when available the number is shown on the graphs.

Jansen's acid clay, the International Vitamin B_1 standard for some years, was supplied to seven pigeons, in doses of 100 mg. daily. All of the pigeons that received this supplement required an unusually long time to become anemic, and presumably this standard is contaminated with the antianemic agents.

Crystalline vitamin B_1 was not available when these studies were begun, but we were able to secure a highly potent vitamin B_1 concentrate. Of twenty-five pigeons which received this material nineteen became severely anemic in periods of 8 to 13 weeks, and the other six became mildly anemic. Just before this investigation was concluded thiamin became available, and it was supplied to five pigeons. For experimental use the crystalline vitamin is vastly preferable to the more or less crude preparations previously available but pigeons which receive this supplement do not become anemic much sooner than do those receiving the crude concentrates just mentioned. Typical histories of anemic pigeons which received the various vitamin B_1 supplements mentioned are summarized in figure 1.

Effect of depletion of vitamin B on development of anemia. The routine procedure to produce pigeon anemia, as previously described, was to deprive the birds of all members of the vitamin B complex. In an effort to determine whether the anemia would develop if the pigeons received an adequate vitamin B_1 supply continuously while consuming the basal diet, four pigeons received 600 gamma daily of the B_1 concentrate from the time they were given ration 1669. The time required to produce anemia in the controls was 7 weeks, but the non-depleted group had not become severely anemic in 20 weeks. During this period they lost weight gradually and continuously. A summary of these observations is shown in table 1.

Relation of other recognized water soluble vitamins to the development of anemia. It seemed entirely possible that the

anemia and loss of weight were due to deficiencies of some of the known members of the vitamin B complex, hence those then available, riboflavin and nicotinic acid, were combined with various vitamin B_1 carriers. Wheat germ oil also was

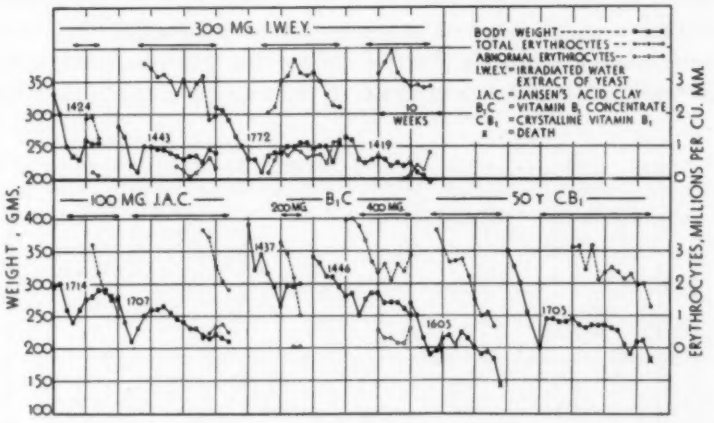

Fig. 1 During the periods indicated by individual arrows the pigeons received vitamin B_1 from various sources of supply. The birds declined in weight and became anemic. Some of the erythrocytes were irregular in outline.

TABLE 1

Average weights and red-cell counts of pigeons not subjected to preliminary depletion of vitamin B_1

EXPERIMENTAL PERIOD	WEIGHT	RED-CELL COUNT
weeks	*gms.*	*millions per cu. mm.*
0	295	3.9 [1]
14	229	2.76
20	208	2.58

[1] The average of all counts made on normal pigeons.

included since it had been shown (Hogan and Richardson, '35) that it heals one type of rat dermatitis. Of the two riboflavin preparations one was crystalline [3], the other was a crude concentrate prepared in the laboratory. The nicotinic acid [4] was

[3] Kindly provided by the Winthrop Chemical Co.

[4] Purchased from the Eastman Kodak Co.

crystalline. In no case did the addition of these substances, or of any combination, have any marked effect on the development of anemia or on the decline in weight. Typical histories of pigeons which received these supplements are shown in figure 2. After these studies were completed other available vitamins were examined but none has been effective.

Anemia not due to fasting. Since the pigeons were subnormal in weight during most of the experimental periods it

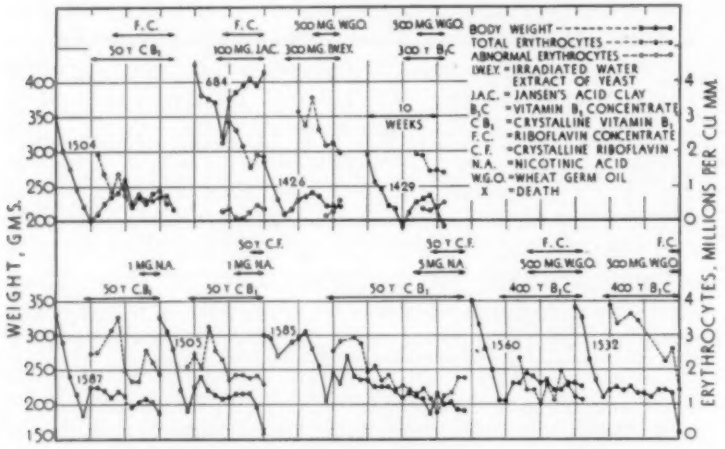

Fig. 2 There was no evidence that the anemia is due to a deficiency of riboflavin, nicotinic acid, or the antidermatitis vitamin of wheat germ oil.

was suggested that the anemia was merely one of the consequences of partial inanition. In our opinion underfeeding does not account for the degree of anemia observed. In the first place many pigeons will make a considerable gain in weight after the administration of vitamin B_1 is begun, although at the same time the red cell counts are declining. Pigeons 1714, 1437, 1446, and 1605 of figure 1, and pigeons 1504 and 684 of figure 2 illustrate this condition. Other examples are shown in the upper half of figure 3, obtained in the course of attempts to find other vitamin concentrates that

would heal the anemia or restore normal weight. These concentrates are not described since none of them was consistently useful. Additional evidence was obtained by using tikitiki, prepared by Wells' method ('21) as a source of vitamin B_1. This material is a potent source of the antianemic agent, but it is deficient in the factors required for weight recovery. It should be pointed out, however, that although in our experience all preparations of tikitiki are active in preventing anemia,

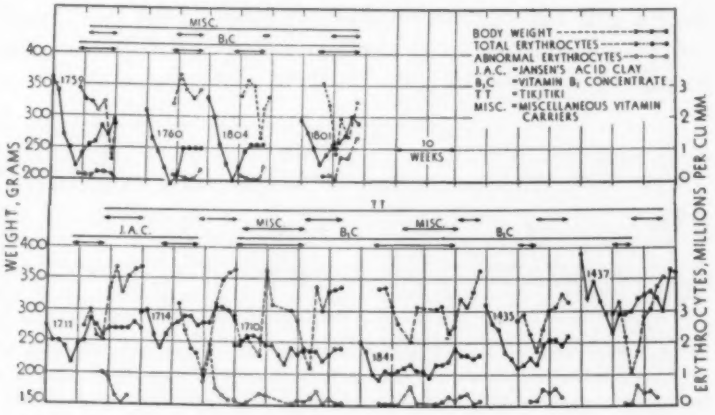

Fig. 3 The upper half of the graph shows that the number of erythrocytes may decline although the pigeons are gaining in weight. Nos. 1759 and 1801 are especially good examples.

The lower half shows that tikitiki permits rapid recovery from anemia, although there may be little or no gain in weight. The periods when the various vitamin carriers were supplied are shown by the individual arrows.

they are variable in their content of the weight recovery factors. On some preparations the gain in weight is negligible, on others the pigeons may recover two-thirds, or more, of the weight lost. Even on these preparations though, the weights will ultimately decline again. Typical responses to tikitiki treatment are shown in the lower half of figure 3.

In order to demonstrate more conclusively that the anemia is not due entirely to a deficient calorie intake, a group of

anemic pigeons was given dried yeast as a curative agent, and at the same time their food intake was restricted so their gain in weight would approximate that of a control group which ate ad libitum, but did not receive any antianemic supplement. As is shown in figure 4 yeast feeding was followed promptly by an increase in the number of erythrocytes, though during this period the red cell counts of the controls steadily declined. It seems clear that the weight maintenance factors are not identical with those that prevent this type of anemia, though they may be interrelated. It is true that Doan, Cunningham, and Sabin ('25) and Barlow ('27) produced a mild anemia by withholding food but their birds were deprived of

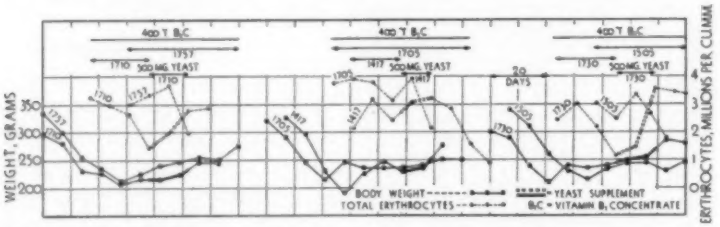

Fig. 4 The number of erythrocytes increased when yeast was consumed, although the food intake of the pigeons was restricted in order to prevent a marked increase in weight. During corresponding periods the number of erythrocytes in the blood of controls continued to decline.

The arrow, which indicates the period during which a vitamin carrier was supplied, bears the same number as the pigeon to which the arrow applies.

vitamins as well as of other nutrients. If pigeons are permitted to consume food ad libitum when yeast is supplied they not only recover from anemia but they also regain normal weight (fig. 5).

Normal erythrocyte count of pigeons. In order to determine the degree of anemia it was necessary to have counts on normal pigeons as a criterion. Ten pigeons were selected at random for these observations, and the counts were made whenever convenient, therefore at irregular intervals. In all, there were forty-six individual red blood-cell counts. The blood was obtained from a wing vein, and diluted 200 times with Tois-

son's solution. The number of red cells per unit volume was rather uniform. The greatest difference in any one individual was 0.78 million per cubic millimeter, and the greatest difference in all counts was 0.86 million. The average was 3.89 million. According to Barlow and Whitehead ('28) the red cells of sixty-eight normal pigeons ranged from 3.78 to 4.52 million per cubic millimeter. In a later publication Barlow ('30) states that the normal erythrocyte count in pigeons is approximately 4 million per cubic millimeter. Kennedy and Climenko ('28-'29) reported an average of 4.3 million for males and 3.6 for females. There is no commonly accepted

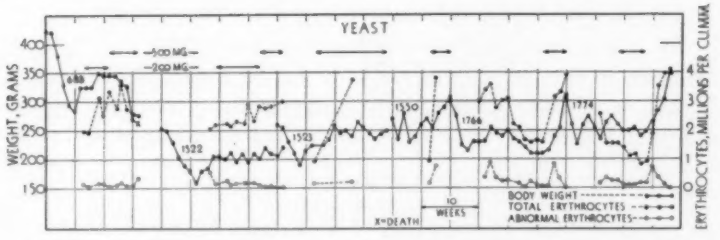

Fig. 5 Pigeons recover from anemia and regain normal weight when supplied with yeast. The response of pigeons 688 and 1522 shows that 200 mg. daily is less than the minimum requirement. The response of the others shows that 500 mg. is enough.

Between the end of the preliminary depletion period and the beginning of yeast feeding various vitamin B_1 carriers were supplied, but these are not shown on the graph.

opinion as to where the dividing line that separates anemic from normal pigeons should be placed but recently we have allowed the number of erythrocytes to fall to between 1.0 to 1.8 million before curative treatment was initiated.

Observations on blood of anemic pigeons. Erythrocytes from a normal and from an anemic pigeon are shown in plate 1. It will be observed that many red cells from the anemic pigeon are abnormal in shape suggesting the sickle-cell anemia occasionally observed in negroes. These deformed cells make their appearance in the blood soon after the number of

erythrocytes begins to decrease, usually in from 5 to 9 weeks after the pigeons have been placed on the experimental ration, no. 1669. The number ranged from 0.10 to 0.30 million per cubic millimeter at the beginning, up to 50% or more of the total number of cells in cases of severe anemia. When the anemic pigeons were given a curative agent, such as tikitiki, the number of deformed cells often increased, and in some cases this increase accounted almost exactly for the total increase in cells. After the first or second week of the curative treatment, however, the number of deformed cells steadily decreased and they usually disappeared entirely from the circulation by the end of the fifth week after the curative treatment was initiated.

As a working hypothesis it is assumed that this type of anemia is due to a deficiency of a previously unrecognized factor, a member of the vitamin B complex. The data do not indicate whether it is a single compound or a mixture.

SUMMARY

1. The procedure followed in producing pigeon anemia is to withhold all members of the vitamin B complex for 4 or 5 weeks, then supply vitamin B_1.

2. The development of anemia was not affected by supplying the pigeons with wheat germ oil, riboflavin, or nicotinic acid.

3. The anemia is not due to partial inanition alone.

4. Some of the erythrocytes of anemic pigeons are abnormal, resembling in some measure the characteristic red corpuscles of sickle-cell anemia.

5. It is suggested as a tentative hypothesis that the anemia is due to a deficiency of a previously unrecognized vitamin.

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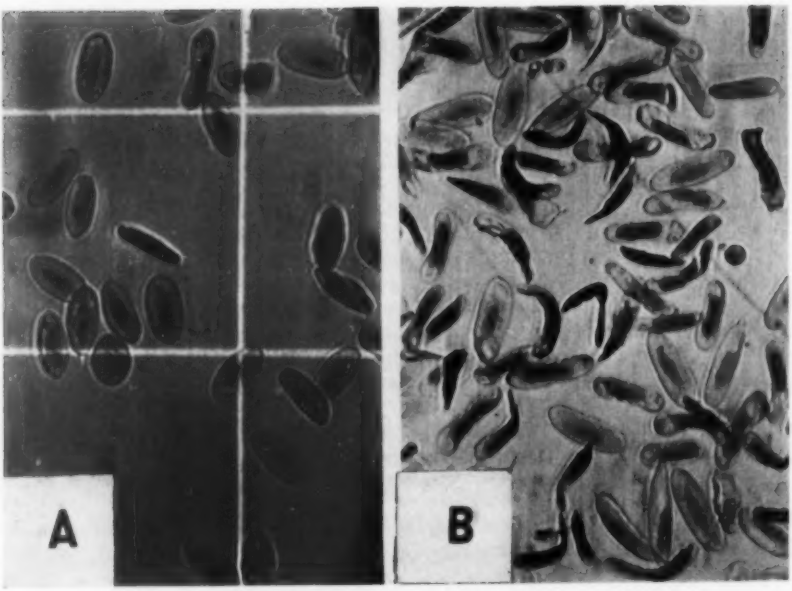

A Erythrocytes of a normal pigeon, 4.08 million per cubic millimeter. Dilution 1 to 200.
B Erythrocytes of an anemic pigeon, 0.74 million per cubic millimeter. Dilution 1 to 20.

THE COMPARATIVE RATE OF ABSORPTION OF SOME NATURAL FATS [1, 2]

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With few exceptions fats have been demonstrated to be almost completely absorbed from the gastrointestinal tract of normal men. In a series of tests on some sixty different types of natural fats by Langworthy ('23) and co-workers, it was found that in practically all cases the coefficient of digestibility exceeded 90%. The chief exceptions noted were in the case of fats having a high melting point such as mutton fat (Langworthy and Holmes, '15), oleostearin (Holmes, '19), and deer fat (Deuel and Holmes, '22), as well as some hydrogenated fats melting from 52° to 60° (Deuel and Holmes, '21) where values as low as 79% were found for the average coefficient of digestibility. McCay and Paul ('38) have noted similar differences in the extent of digestion of the higher melting fats in guinea pigs.

On the other hand, very few studies have been carried out on the rate of absorption of the different fats to ascertain the factors which might alter this property. Steenbock, Irwin and Weber ('36), employing a technic described earlier by Irwin, Steenbock and Templin ('36) reported that lard, hydrogenated cottonseed oil, and butter were absorbed to the extent of about 60% in a 6-hour period after 1.5 cc. of the fat was administered to rats, while linseed oil disappeared

[1] Some of this work was made possible by a research grant from The Best Foods, Inc.

[2] Some of these results were reported at the meeting of the Institute of Nutrition at New Orleans, March 13, 1940.

at a somewhat more rapid rate. On the other hand no sex differences were noted nor were the authors able to correlate the rate of absorption with body weight, surface area, or the length of the intestine.

In the present study the comparative rates of absorption of several fats differing in chemical nature have been investigated. In the first place a fat having a low saponification number (rape seed oil) has been compared with several fats having high saponification values (butter fat and coconut oil). Secondly, a comparison has been made of the rate of absorption of a hydrogenated cottonseed oil with a "wintered" cottonseed oil from which the tristearin had been largely removed. The hydrogenated cottonseed oil was fed in different size doses and to rats which were widely divergent in body weight.

EXPERIMENTAL

The fats were fed by stomach tube to rats previously fasted for 48 hours, the material being measured in tuberculin syringes of 1 or 2 cc. capacity which were connected with the stomach tube. No anesthesia was employed.

The fat could be measured with considerable accuracy by this technic. The results (in milligrams) of successive unselected tests when 1 cc. of the melted fat was discharged into flasks and weighed are as follows: rape seed oil (910, 913, 914), butter fat (908, 907, 910), coconut oil (920, 918, 919), tricaprin (928, 927, 928). When 1.5 cc. quantities were employed, the values for some other fats are as follows: triacetin (1702, 1705, 1713, 1695), tripropionin (1601, 1605, 1605, 1605, 1599) and tributyrin (1540, 1558, 1548, 1554, 1557).

After periods of 3, 4½, or 6 hours the rats were anesthetized with amytal, the intact gastrointestinal tract was removed, and the fats remaining in the gut removed by the procedure of Deuel, Hallman and Quon ('39). In the experiments recorded in tables 5 and 6, the fat was recovered by a modified procedure which was equally as satisfactory. The stomach and entire gut including the caecum and colon were removed intact. In the modified technic, after inserting a blunt needle

into the esophagus, 70 cc. of diethyl ether was flushed through the gastrointestinal tract instead of water and petroleum ether as used in the earlier method. This "washing" was dried with anhydrous Na_2SO_4 overnight; the extract was then filtered into weighed Bailey-Walker fat flasks after which the residue was washed several times with generous quantities of ether. After removal of the ether, the fat remaining in the flask was weighed.

A correction was applied for the recovered fat by subtraction of the quantity of ether-soluble material which was removable from the gastrointestinal tracts of rats fasted for a similar period but fed no fat (table 1). A further correction

TABLE 1

The average lipid content of the gastrointestinal tract of rats previously fasted for 2 days

PROCEDURE AND SOLVENT	NUMBER OF EXPERIMENTS	SEX	BODY WEIGHT	LIPID		
				Maximum	Minimum	Average
			gm.	*mg.*	*mg.*	*mg.*
I, Petroleum ether	20	M	225	23	0	12.5
I, Petroleum ether	9	F	150	5	1	3.0
II, Diethyl ether	11	M	214	43	9	24.9
II, Diethyl ether	10	M	75	50	16	28.9

is made for the extent of recovery of the fat by these procedures based on the values obtained when known amounts of fat are given and the gastrointestinal tract removed immediately (table 2). The results of the control tests are summarized in table 1.

In order to determine the extent of recovery of fats from the gastrointestinal tract by the use of procedure I (warm water followed by petroleum ether) and procedure II (diethyl ether alone), experiments were carried out in which known quantities of fat were administered by stomach tube to fasted rats followed by the immediate removal of the gastrointestinal tract and the flushing out of their contents with the fluid used. The recovery is expressed as the per cent of the original fat given after correction for the control lipid values of fasted

rats. Although a small variation exists in the quantity of material extractable from the gastrointestinal tract of the fasted control rats when petroleum ether and diethyl ether are used, it is apparent that these differences are concerned with their solvent action on metabolic lipid residues rather than on the triglycerides. These results are summarized in table 2.

The rats used in the tests were obtained from our stock colony. They had previously been fed the stock diet described elsewhere (Butts, Cutler, Hallman and Deuel, '35).

TABLE 2

The recovery of various fats from the gastrointestinal tract of rats killed immediately after administration of the fat by stomach tube

FAT FED	PROCEDURE USED	NUMBER OF EXPERIMENTS	SEX	FAT FED	FAT RECOVERED		
					Total	Corrected	Per cent
				mg.	*mg.*	*mg.*	
Cottonseed oil (wintered)	I	11	M	908	876	864	95.2
	I	15	F	1360	1294	1291	94.9
Cottonseed oil (hydrogenated)	I	13	M	809	764	752	92.6
	I	9	M	2835	2726	2714	95.7
	II	12	M	809	782	757	93.6
Rape-seed oil	I	10	M	912	801	789	86.5
	I	10	M	3008	2723	2711	90.1
Linseed oil	I	6	F	928	888	885	95.3
	II	12	M	928	917	892	96.1

In most cases the animals were 3 to 5 months of age with the exception of those used in the experiments recorded in table 5 where the large males were approximately 7 months of age while the young rats averaged about 6 weeks. The weight of the rats was determined after a 48-hour fast. From this value surface area was calculated by the formula of Lee ('29).

The "wintered" cottonseed oil and the hydrogenated cottonseed fat were commercial products obtained on the open market. The hydrogenated cottonseed oil was fed in its

original form as a homogenous mixture with skimmed milk which contained 0.809 gm. of fat per cubic centimeter. The other fats were obtained from known sources. Table 3 gives the constants of the fats used. All were liquid at body temperature. The constants of the fats studied were within the values usually assigned.

RESULTS

In the first series of tests the rate of absorption of the various fats when fed in amounts of 1 cc. was determined in experiments of 3, 4½ and 6 hours duration. This quantity of fat was used because diarrhea frequently occurred when larger doses were employed. After the administration of the fats,

TABLE 3

The physical and chemical constants of the fats used[1]

TYPE OF FAT	SPECIFIC GRAVITY	SAPONIFICATION NUMBER	IODINE NUMBER
Butter fat	0.908	230	28.2
Hydrogenated cottonseed oil	0.903	197	67.6
Wintered cottonseed oil	0.907	190	93.2
Coconut oil	0.919	249	13.0
Rape-seed oil	0.912	175	87.2

[1] These constants were determined by A. Reifman.

the rats were placed in individual cages and the experiments discarded if any evidence of diarrhea was noted. The summaries of the results are included in table 4.

In order to determine whether any correlation exists between the size of the animal and the quantity of fat which it is able to digest, the experiments summarized in table 5 were carried out.

The comparisons in the rate of absorption of the same fat when given in increased amounts to rats of approximately the same size are recorded in table 6.

TABLE 4

The absorption of various fats by rats following the administration of 1 cc.

LENGTH OF PERIOD	SEX	NUMBER OF TESTS	BODY WEIGHT	SURFACE AREA	FAT ABSORBED			NUMBER OF CASES OF DIARRHEA
					Per cent of fat fed	Per 100 gm. per hour [1]	Per 100 sq.cm. per hour [1]	
Hydrogenated cottonseed oil (809 mg.)								
hours			*gm.*	*sq.cm.*				
3	M	10	186	287	47.7	69.6±5.2	44.5±3.3	2
4.5 [2]	M	9	159	262	81.4	95.1±5.6	56.9±3.1	
6 [2]	M	10	162	265	90.4	76.0±3.6	46.1±1.9	
6	F	10	152	254	75.5	67.1±2.9	39.7±1.8	
Butter fat (908 mg.)								
3	M	9	174	273	44.7	80.4±4.8	49.6±2.7	5
4.5	M	10	168	268	63.2	79.3±6.4	43.2±3.2	
6 [2]	M	12	197	297	81.6	65.3±4.6	42.6±2.1	7
3	F	6	160	262	45.7	86.4±8.5	52.6±5.1	12
6	F	5	171	273	74.5	67.4±4.7	41.6±2.1	9
Rape-seed oil (912 mg.)								
3	M	11	204	303	37.5	59.4±5.6	38.5±3.0	9
4.5	M	11	175	277	55.8	65.1±3.7	40.7±2.1	1
6	M	11	195	296	58.2	46.1±3.2	30.0±1.8	1
3	F	5	158	261	32.5	62.5±2.5	37.0±1.5	5
6	F	10	146	250	43.4	44.8±2.9	26.2±1.9	
Wintered cottonseed oil (907 mg.)								
3	M	10	210	310	41.8	59.3±3.9	39.8±2.9	3
4.5	M	9	193	293	69.3	74.2±4.2	47.9±2.3	
6	M	10	175	277	72.4	63.5±3.4	39.7±1.5	
6	F	8	146	249	71.5	72.8±3.3	43.3±1.3	
Coconut oil (919 mg.)								
3	M	8	147	249	34.7	71.4±7.2	42.0±4.5	18
4.5	M	11	158	260	59.5	78.2±3.6	46.7±1.8	14
6	M	7	171	274	76.7	70.0±4.5	43.2±2.0	14

[1] Including the standard error of the mean calculated as follows:

$$\text{S. E. M} = \sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$$

where d is the deviation from the mean and n is the number of observations.

[2] Including experiments where practically complete absorption obtained. These must be considered minimum values.

TABLE 5

The effect of size on the rate of absorption of hydrogenated cottonseed oil by rats during a 3-hour period

GROUP	NUMBER OF EXPERIMENTS	AVERAGE BODY WEIGHT	AVERAGE SURFACE AREA	FAT FED IN MILLIGRAMS		FAT ABSORBED			
				Total	Per 100 sq.cm.	Total	Per cent of fat fed	Per 100 gm. per hour [1]	Per 100 sq.cm. per hour [1]
Male rats									
		gm.	*sq.cm.*			*mg.*		*mg.*	*mg.*
I	23	270	360	1087	300	469	43.2	57.8 ± 2.4	43.3 ± 1.8
II	19	65	153	1101	740	254	23.1	134.5 ± 9.7	56.1 ± 3.4
III	15	74	165	502	300	256	51.0	117.9 ± 7.4	53.8 ± 2.6
Female rats									
IV	9	150	253	989	391	289	29.2	64.2 ± 5.4	38.1 ± 3.2
V	16	64	150	452	300	192	43.5	105.2 ± 5.9	43.7 ± 2.1

[1] Including standard error of mean.

TABLE 6

The effect of the amount of fat fed on the rate of absorption in fasting male rats

GROUP	NUMBER OF EXPERIMENTS	AVERAGE BODY WEIGHT	AVERAGE SURFACE AREA	FAT FED IN MILLIGRAMS		FAT ABSORBED			
				Total	Per 100 sq.cm.	Total	Per cent of fat fed	Per 100 gm. per hour [1]	Per 100 sq.cm. per hour [1]
Hydrogenated cottonseed oil									
		gm.	*sq.cm.*			*mg.*		*mg.*	*mg.*
VI	15	266	357	486	136	291	60.1	36.8 ± 1.6	27.2 ± 1.1
I	23	270	360	1087	300	469	43.2	57.8 ± 2.4	43.3 ± 1.8
VII	10	198	299	940	300	433	46.1	73.9 ± 6.5	48.4 ± 3.9
VIII	13	203	304	1824	600	401	22.0	65.5 ± 3.9	43.8 ± 2.7
IX	11	204	304	2668	900	514	19.2	84.2 ± 3.4	56.3 ± 2.3
III	15	74	165	502	300	252	50.2	115.7 ± 7.5	50.2 ± 3.1
II	19	65	153	1101	740	240	22.8	127.7 ± 9.8	53.1 ± 3.5
Rape-seed oil									
X	15	191	293	882	300	295	33.4	52.8 ± 5.1	34.0 ± 3.1
XI	8	176	278	2154	900	373	17.3	71.4 ± 8.8	44.7 ± 5.0

[1] Including standard error of mean.

DISCUSSION

The differences in the rate of absorption of the different natural fats investigated are not great in spite of a marked variation in the nature of their fatty acid components. In most cases the averages are between 40 and 55 mg. per 100 sq.cm. per hour.

Rape-seed oil consistently seemed to be absorbed most slowly. This is noted in the 3-hour tests although in the experiments continued for 6 hours the variations are extremely pronounced. At the latter period the rates were 30.0 and 26.2 mg. per 100 sq.cm. per hour for the animals receiving rape-seed oil. It seems possible that the rate of hydrolysis of the longer fatty acids in rape-seed oil or their absorption may be even slower than these results indicate. The higher values during the shorter periods (although still the lowest in the 3-hour period) may result from the normal rate of digestion and absorption of the component palmitic, stearic and oleic acids which are in the molecule. After the disappearance of these, the rate falls markedly because of the inability of the higher chain triglycerides to be as readily utilized.

In the experiments with individual fats, it was our experience that large rats absorbed greater quantities during a given interval than small animals. Since this is the case, the rate of absorption of the fat (as expressed in per cent of the administered fat which disappeared) would vary with the size of the rats employed in the tests. If the animals used in comparison of various fats were of approximately uniform weight (as in the experiments of Steenbock et al.), then comparison on such a basis would be valid; on the other hand, if a wide variation in the average weight obtained, such a method of comparison might be open to serious errors. In order to determine whether the rate of absorption might be correlated with body weight or with surface area, studies were made on the rate of absorption in rats of widely varying size. When hydrogenated cottonseed oil was administered in a dose of approximately 1100 mg. to large male rats (group

I), 469 mg. or 43.2% of the total was absorbed; when the same amount was given to small male rats (group II), only 254 mg. or 23.1% of the total fat administered disappeared in a similar interval. Although the results noted when the comparison is made on the basis of body weight (57.8 mg. per 100 gm. per hour for the large rats and 134.5 mg. for the small ones) are equally as variable, the values show much greater uniformity when based on surface area (43.3 and 56.1 mg. per 100 sq.cm. per hour for the large and small rats respectively). These values become practically identical if allowance is made for differences in metabolic activity of the larger and smaller animals. According to Horst, Mendel and Benedict ('34), the basal metabolism for male rats 7 months in age amounts to 755 Calories per square meter per day while that of rats 7 weeks old is 923 Calories. When the rates of absorption of the small rats (whose experiments are recorded in table 5) are multiplied by a correction figure of 0.818 (obtained by dividing 755 by 923), the corrected rate of absorption of the small rats (groups II and III) becomes 45.9 and 44.0 mg. respectively compared with the level of 43.3 for the large animals.

When the doses of the fat administered to the large and small rats were uniform on the surface area basis (i.e., 300 mg. per 100 sq.cm.), then the same quantitative differences in amounts absorbed are still maintained as in groups I and II, but the per cents absorbed of the original quantity fed more nearly approximate each other (43.2% in the large rats (group I), and 51.0% in small rats (group III)). The uniformity in absorption expressed on the surface area is equally as satisfactory, the corrected values being 43.3 and 44.0 mg. per 100 sq.cm. per hour respectively. Similar results are to be noted in the total amount of fat digested by large and small female rats when fed at the level of 300 mg. per 100 sq.cm., but the absorption calculated on the basis of surface area is uniform (38.1 and 43.7 mg. per 100 sq.cm. per hour for the large and small rats respectively).

The per cent of fat absorbed also is inversely proportional to the quantity of fat administered. These results are also in harmony with the report of Steenbock, Irwin and Weber. However, the total quantity of fat which disappears apparently does vary to some extent with the dose given. The greatest alteration is noted in group VI (table 6) where the quantity absorbed was only 291 mg. when fed at the low level of 136 mg. per 100 sq.cm. When the dose was increased to 300 mg. per 100 sq.cm., the quantity absorbed by rats of similar size was 469 mg. in a like interval. Practically no variations could be noted in the rate of absorption when the doses varied between 300 and 740 mg. per 100 sq.cm., although the highest rate of absorption of hydrogenated cottonseed oil (group IX) and of rape-seed oil (group XI) was found when the dose was increased to 900 mg. per square centimeters. Although the dosage of the fats in the experiments recorded in table 4 was not on the basis of surface area, the variation in quantity administered when calculated on that basis was small (300 to 400 mg. per square centimeter) because of the uniformity in size of the rats. The variations in the absorption rate are not to be attributed to different amounts fed.

SUMMARY

A procedure for recovery of fat from the gastrointestinal tract is described in which the average recovery for various fats in eight of the nine series of tests exceeds 90%.

When the same quantities of hydrogenated cottonseed oil were administered to rats of widely varying weights, the amount absorbed is considerably greater in the large rats. However, most uniform results are obtained when the comparison of absorption is made on the basis of body surface area.

In rats of similar size, the quantity of fat absorbed increases somewhat when increased amounts are fed.

No consistent differences in the rate of absorption of hydrogenated cottonseed oil, "wintered" cottonseed oil, butter

fat, or coconut oil were noted. In most cases the rate of absorption lay between 40 and 50 mg. per 100 sq.cm. per hour in experiments of 3, 4½ and 6 hours duration. However, the absorption of rape-seed oil was somewhat slower, the maximum differences being noted in the 6-hour tests where values of 30.0 and 26.2 mg. were obtained on groups of male and female rats respectively.

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THE RATE OF ABSORPTION OF SYNTHETIC TRIGLYCERIDES IN THE RAT [1, 2]

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The physiological behavior of the triglycerides of the lower fatty acids differs in certain respects from that of the higher triglycerides such as tripalmitin and tristearin. In the first place the fatty acids formed on hydrolysis of the lower triglycerides (up to triheptylin) are to some extent water soluble although the latter two acids (caproic and heptylic) are only slightly so (0.4 gm. and 0.24 gm. per 100 cc.; Hodgman, '37). Under such conditions the presence of bile salts is probably not necessary for their absorption. Moreover, there is considerable evidence that no fats lower than tricaprylin can be deposited as such in the tissues even when fed in fairly large doses (Eckstein, '29 a, '29 b). Lastly, triacetin, tripropionin, tributyrin, and trivalerin all have higher specific gravities than water. Although there is no concrete evidence that this may alter their physiological behavior, we have found that extraction procedures suitable for recovery of the fats having a specific gravity less than one are unsatisfactory when applied to these heavier fats.

The lower synthetic triglycerides apparently are readily digested although no studies are available as to their relative rates in vivo when compared with the natural fats or when compared with each other. Weinstein and Wynne ('36) showed that they were readily attacked by pancreatic lipase in vitro.

[1] Some of this work was assisted by a research grant from The Best Foods, Inc.

[2] These data were presented before the Institute of Nutrition at the meeting in New Orleans.

Tripropionin was hydrolyzed most rapidly followed in order by tributyrin, tricaproin, triacetin, and trivalerin. Using a different procedure, Balls, Matlack and Tucker ('37) found that temperature had a marked effect on the rate of hydrolysis of the lower triglycerides. At 0°C. there was a progressive rise in the speed of hydrolysis with the lengthening of the chain which reached a maximum with triheptylin. At this temperature the hydrolysis of the glycerides above tricaprin was practically blank even after a period of 144 hours. On the other hand at 40°C. the maximum speed of hydrolysis occurred with trilaurin and complete hydrolysis was noted within a 3-hour period while the shorter chain triglycerides were more slowly broken down, the minimum value of 28.4% being obtained for triacetin. The only irregularity in the progressive decrease in rate of hydrolysis with the varying length of the carbon chain under the latter conditions was with respect to triisovalerin which was more slowly hydrolyzed than any fat except triacetin. Deuel, Butts, Blunden, Cutler and Knott ('37) also demonstrated indirectly the differences in metabolism of the synthetic fats with fatty acids of eight carbons or less when compared with the natural fats. Since the former could not be stored (possibly also could not be resynthesized in the intestinal mucosa), the glycerol from them is available for immediate conversion to liver glycogen. With the natural fats which contained exclusively fatty acids capable of deposition in the tissues as neutral fat, no glycerol was available for such conversion to carbohydrate. As might be expected, the triglycerides composed of odd-chain fatty acids (tripropionin, trivalerin, and triheptylin) gave rise to greater quantities of liver glycogen than those composed of even-chain fatty acids because the fatty acid portion of their molecules was also glycogenic.

In view of these variations in the in vitro studies, it seemed of importance to ascertain whether similar differences might be noted when the absorptions were compared in vivo. In the present study the extent of digestion and absorption of the synthetic triglycerides from triacetin to tricaprylin as well

as triisovalerin and trilaurin has been studied in rats over a 3-hour period when they were fed in doses equivalent to 300 mg. per 100 sq.cm. of surface area.

METHODS

The rate of absorption of the synthetic triglycerides has been determined by the procedure described earlier (Deuel, Hallman and Leonard, '40). Since some of the fats had specific gravities greater than unity, the earlier procedure which involved their extraction by petroleum ether from a water extract could not be employed. However, by extraction of the

TABLE 1

The recovery of various synthetic fats from the gastrointestinal tracts of rats killed immediately after administration of the fat

FAT FED	NO. OF EXPTS.	AVERAGE WEIGHT	FAT GIVEN	FAT RECOVERED		
				Total	Corrected for blank	Per cent [1]
		gm.	*mg.*	*mg.*	*mg.*	
Triacetin	12	220	1144	1011	987	86.3±0.9
Tripropionin	12	192	1072	1002	990	92.2±0.5
Tributyrin	12	168	1040	991	963	92.7±0.5
Trivalerin	12	228	1020	912	887	87.2±0.7
Triisovalerin	10	213	1002	980	955	95.4±0.2
Tricaproin	11	234	968	899	874	90.6±0.9
Triheptylin	13	223	960	906	880	91.9±0.4
Tricaprylin	9	233	914	886	861	94.2±0.6
Tricaprin	9	176	696 [2]	695	670	96.5±0.4
Trilaurin	11	209	918	909	884	96.1±0.5

[1] Including standard error of mean.

[2] Only 0.75 cc. administered.

gastrointestinal tract with diethyl ether alone without water, the recovery of the fat could be accomplished satisfactorily. The results when 1 cc. quantities of the fats were given by stomach tube and the gastrointestinal tracts immediately removed and flushed with ether are recorded in table 1.

The fats used were obtained from the Eastman Kodak Co.[3] Their purity was established by the determination of the

[3] One of the samples of trilaurin used was kindly furnished us by Dr. Samuel Lepkovsky.

saponification numbers. Other experimental procedures used were similar to those employed in the earlier work (Deuel, Hallman and Leonard, '40).

RESULTS

The results of the various experiments are summarized in table 2.

TABLE 2

The absorption of various synthetic fats from the gastrointestinal tract of fasting male rats during a 3-hour period

FAT FED	NO. OF EXPTS.	BODY WEIGHT	SURFACE AREA	ABSORPTION IN MG. PER HR.[1]	
				Per 100 gm.	Per 100 sq.cm.
		gm.	*sq.cm.*		
Triacetin	12	205	305	102.3±2.9	68.1±1.4
Tripropionin	12	162	264	51.6±3.4	31.4±2.1
Tributyrin	10	190	291	102.3±6.3	65.0±2.5
Trivalerin	9	190	291	50,5±2.9	32.9±2.3
Triisovalerin	6	194	295	69.6±3.3	45.7±2.5
Tricaproin	9	192	292	83.8±2.3	54.5±1.5
Triheptylin	10	207	306	42.0±2.6	28.0±1.6
Tricaprylin	8	226	324	66.5±6.2	45.9±4.1
Trilaurin[2]	5	230	328	31.5	21.9

[1] Including standard error of mean.
[2] No statistical treatment.

DISCUSSION

The neutral fats composed of the lower fatty acids having an even number of carbon atoms are readily broken down and absorbed in the rat. Triacetin and tributyrin, having rates of 68 and 65 mg. per 100 sq.cm. per hour, are more rapidly absorbed than any of the natural fats investigated. Tricaproin is somewhat slower while tricaprylin is absorbed at a level which compares with most of the natural fats.

No successful experiments were completed with tricaprin because in each of twenty experiments a violent diarrhea ensued. The slow absorption of trilaurin is probably to be traced to its high melting point. The fat solidified in a hard mass in the stomach of the rats. Had it been dissolved in a fat liquid at body temperature, the action of the pancreatic

lipase would probably have been facilitated with the result that the absorption would have compared favorably with that of the natural fats.

The fats having fatty acids with an uneven number of carbon atoms were absorbed at 50% of the rate of the corresponding even-chain fats. Thus, the rates obtained for tripropionin, trivalerin and triheptylin of 31, 33 and 28 mg. per 100 sq.cm. per hour respectively are smaller than those for any of the natural or synthetic fats studied for 3-hour periods.

The results yielded by the in vivo studies differ from those obtained by in vitro digestion of similar fats with pancreatic lipase. Not only were no differences observed in the latter tests between odd and even-chain fats but it was also noted that triacetin was least readily broken down. In the present (in vivo) experiments just the opposite results were noted, i.e., that triacetin was the most readily digested of any of the fats studied. Balls, Matlack and Tucker noted that triisovalerin was one of the most slowly digested triglycerides; in the present series this interesting fat was digested fairly rapidly at a rate similar to that of tributyrin or tricaproin rather than that of trivalerin.

It is possible that the rate of in vivo hydrolysis is similar to that observed in vitro, but that the differences noted between the results obtained in the two types of experiments may be related to variations in the rate of absorption of the acids resulting from the digestion. As far as the authors are aware, no comparative studies on the rate of the absorption of the short-chain fatty acids have been made although the ready glycogen formation after propionic, valeric and heptylic acids (Deuel, Butts, Hallman and Cutler, '35) would seem to indicate that the absorption is a fairly rapid one. Experiments are under way to compare the rate of absorption of the short-chain fatty acids.

SUMMARY

Triacetin and tributyrin were the most rapidly absorbed fats of any natural or synthetic fats studied; tricaproin and

tricaprylin disappeared somewhat more slowly from the alimentary tract of the rat. Trilaurin was only slowly removed from the gastrointestinal tract presumably because of its high melting point which caused its solidification.

The neutral fats composed of odd-chain fatty acids (tripropionin, trivalerin, and triheptylin) were absorbed at a rate of 50% or less of that characteristic of the corresponding even-chain fats. Triisovalerin was absorbed at a rate which compared with the even-chain fats.

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THE MINIMUM REQUIREMENT OF RIBOFLAVIN FOR THE GROWING PIG [1]

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TWO FIGURES

(Received for publication May 6, 1940)

INTRODUCTION

The necessity of riboflavin in the nutrition of the pig was demonstrated by Hughes ('39). The present investigation includes two experiments conducted to determine the minimum requirement of riboflavin for growing swine; the first was started in November, 1938, and the second concluded in August, 1939.

EXPERIMENTAL

In the summer of 1938, synthetic riboflavin was available and an effort to determine the minimum requirement of riboflavin was begun that fall.

The basal diet consisted of cane sugar 79%, casein (purified) 15%, lard 1%, rice bran filtrate 2%, salt mixture 3%, cod liver oil 10 cc. per pig weekly, thiamin 4 mg. and nicotinic acid 15 mg. per 100 pounds of pig daily. The cod liver oil was given by mouth once each week, while the thiamin and nicotinic acid were dissolved in distilled water and given to each pig by mouth twice weekly.

The casein was purified by the method used previously (Hughes, '39). The rice bran filtrate was prepared by the method of Lepkovsky and Jukes ('35). The product was

[1] The experimental work reported in this paper became cooperative with the United States Bureau of Animal Industry, July 1, 1938.

treated with sufficient fuller's earth to remove the riboflavin and thiamin.

The sugar, casein, lard (melted before being mixed), rice bran filtrate, and salt mixture were mixed weekly and placed in new receptacles (garbage cans) which were paper lined.[2] The salt mixture was the same as that used previously (Hughes, '38).

Five groups of pigs (weighing about 80 pounds at the beginning of the experiment) with five in each lot were fed the following diets beginning December 12, 1938:

Diet 1—Basal (no riboflavin).
Diet 2—Basal + 1 mg. riboflavin per 100 pounds of pig per day.
Diet 3—Basal + 3 mg. riboflavin per 100 pounds of pig per day.
Diet 4—Basal + 5 mg. riboflavin per 100 pounds of pig per day.
Diet 5—Basal with no lard (1 pound sugar replaced 1 pound of lard) + 4 mg. riboflavin per 100 pounds of pig per day.

The pigs used were uniform in size and age. They were fed and watered in steel troughs. Their quarters were similar in size and shape, having concrete floors and wooden partitions. They had free access to inside and outside pens.

The pigs in all lots gained very slowly; the average per pig in all groups at the end of 38 days was only about 10 pounds each. One pig in the control group (diet 1) vomited 3 days after the experiment began, another was unable to get up at the end of 20 days and died the same day. All the pigs chewed at the fences, and became very thin. At the end of the first 40-day period one pig had died, and the others were either down and unable to rise or were very unsteady on their legs. Two pigs were given 5 mg. of thiamin in a vein of the ear on January 20. It seemed to be of some benefit to one pig, this animal becoming more active, but not to the other. Two days later another pig was given 20 mg. in the ear with no perceptible change. The fourth pig was given 10 cc. of wheat germ

[2] The sugar was secured from the California-Hawaiian Sugar Company; the casein from Golden State Company, Ltd.; the lard from Cudahy and Company; the rice bran filtrate from Vitab Products, Inc.; the thiamin and riboflavin from Merck and Company; the nicotinic acid from the Eastman Kodak Company and the cod liver oil used was Squibb's tested oil.

oil orally on January 29 with no apparent success. These pigs (lot 1—basal) gained an average of 21.3 pounds in 73 days or had an average daily gain of 0.3 pounds.

One pig in lot 2 died on the fifty-fifth day of the experiment and three others were down or had extreme difficulty in getting up. Stiffness, unsteady gaits or trembling were apparent in these pigs throughout the major part of the experiment.

In group 3 the conditions were like those of lot 2. There was one death; the pigs were all unsteady on their feet and two were down and not able to rise.

There was one death in group 4, the same amount of stiffness as in the former groups and unsteady gaits. Three pigs were down and unable to get up.

Group 5 were not fed lard. The pigs behaved like those in the former groups, except that they were more persistent in chewing the fences. One pig was down and another died the last day of the experiment. The results of this experiment were not definite. The pigs fed the basal ration (diet 1) gained more slowly than the rest; however, the pigs in the other lots were not normal.

Because the problem had not been solved another experiment was conducted. It was similar in many respects yet differed in some very important particulars. Increasing the thiamin content of the ration, the addition of wheat germ oil and giving ascorbic acid did not materially change the effects in the early experiment. Believing that we were dealing with a multiple deficiency in the first test, it was logical to increase the rice bran filtrate in the second experiment. The lard in the first test did not materially effect the results so it was left out in the second.

The following rations were fed in the second experiment:

Lot 1b—Basal ration, cane sugar 77%, casein (purified) 15%, rice bran filtrate 5%, salt mixture 3%, cod liver oil 10 cc. per pig weekly, thiamin 6 mg. and nicotinic acid 15 mg. per 100 pounds of pig per day.

Lot 2b—Basal ration + 1 mg. riboflavin per 100 pounds of pig per day.

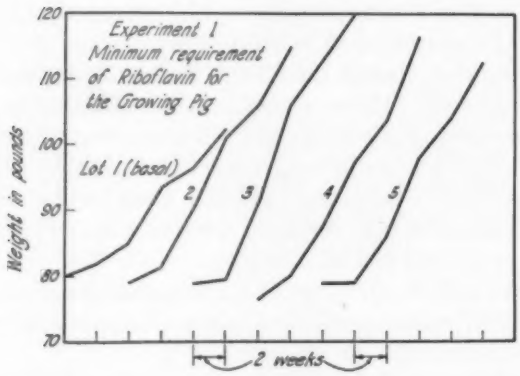

Fig. 1 Growth curves of pigs in experiment 1. The pigs in lot 1 (fed no riboflavin) gained more slowly than those in the other lots. The average age of the pigs at the beginning was about 85 days.

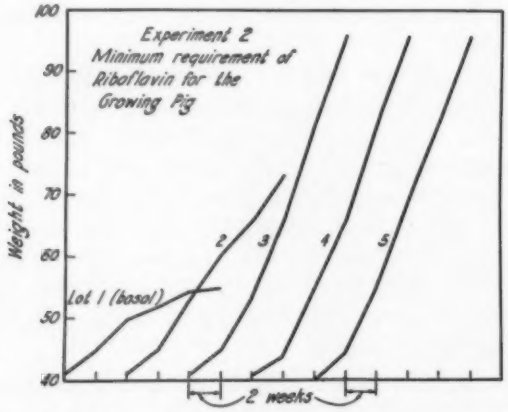

Fig. 2 Pigs on the basal diet gained very slowly; those fed 1 mg. riboflavin daily gained more rapidly (lot 2b); while those in the other groups gained faster than those in group 2b and at the same rate. The average age of the pigs at the beginning of the experiment was about 55 days.

Lot 3b—Basal ration + 3 mg. of riboflavin per 100 pounds of pig per day.
Lot 4b—Basal ration (12 mg. of thiamin instead of 6 mg.) + 3 mg. of riboflavin per 100 pounds of pig per day.
Lot 5b—Basal ration + 6 mg. of riboflavin per 100 pounds of pig per day.

There were five pigs in each group in the second experiment, which were fed in the same pens as those of the first experiment. The thiamin, nicotinic acid, and riboflavin were put in water solution and mixed with the feed each week.

The results of this experiment are graphically represented in figure 2.

The graphs show very slow growth in group 1b, somewhat better growth in lot 2b where 1 mg. of riboflavin was fed. The rate of growth in the other lots was more rapid and practically identical.

DISCUSSION

While the results of the first experiment show clearly the need for riboflavin (see fig. 1, lot 1), there was practically no difference in the growth rates of those in groups 2, 3, 4 and 5. Because there was so much stiffness, staggering gaits, paralysis and some deaths, it was believed that we were probably dealing with a double deficiency in this experiment. In the second experiment the rice bran filtrate was increased to 5% of the diet. This proved to be very beneficial and showed the importance of the filtrate factor, vitamin B_6, or other factors in the rice bran preparation in the nutrition of the pig.

In the second experiment the results are clear cut. The minimum requirement of riboflavin as determined under the conditions of this experiment lies between 1 and 3 mg. of riboflavin (fig. 2) for each 100 pounds of pig per day. The rate of growth for the basal group was extremely slow, and while the growth rate for group 2b, fed 1 mg. of riboflavin, was superior to that of the basal, it was much less than those of groups 3b, 4b and 5b. Increasing the thiamin in group 4b to 12 mg. per 100 pounds was without effect. Doubling the riboflavin given pigs in group 5b over those in groups 3b and 4b was also without effect.

SUMMARY

From the results of these studies, using purified diets, it is concluded that the minimum requirement of riboflavin for the young growing pig lies between 1 and 3 mg. per 100 pounds of pig daily.

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THE MINIMUM REQUIREMENT OF THIAMIN FOR THE GROWING PIG [1]

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ONE FIGURE

(Received for publication May 6, 1940)

INTRODUCTION

Thiamin chloride hydrochloride was given to pigs fed purified diets by Chick et al. ('38), Wintrobe and co-workers ('38) and Hughes ('38). That thiamin is necessary for normal growth and well being was demonstrated by Hughes ('39). In the fall of 1939 it was decided to determine, if possible, the minimum requirement of this factor for the growing pig. Some evidence had been obtained that the requirement was less than 6 mg. per 100 pounds of pig daily (Hughes, '39).

EXPERIMENTAL

The basal diet in this study was the same as that used in determining the minimum requirement of riboflavin for the pig except that thiamin, and not riboflavin, was fed at varying levels. Four groups of pigs weighing between 60 and 65 pounds at the beginning of the experiment were fed the following diets:

Group 1—Basal ration, composed of cane sugar 77%, purified casein 15%, salt mixture 3%, rice bran filtrate 5%, cod liver oil 5 to 10 cc. per head weekly, nicotinic acid 15 mg. and riboflavin 3 mg. per 100 pounds of pig daily.

[1] The experimental work reported in this paper became cooperative with the United States Bureau of Animal Industry, July 1, 1938.

Group 2—Basal + 1 mg. thiamin per 100 pounds of pig daily.
Group 3—Basal + 3 mg. thiamin per 100 pounds of pig daily.
Group 4—Basal + 6 mg. thiamin per 100 pounds of pig daily.

The experiment began December 12, 1939, and continued into February, 1940. There were five vigorous uniform pigs in each lot. The cod liver oil was given by mouth each week while the riboflavin, nicotinic acid and thiamin were put in water solution and mixed with the feed at the beginning of

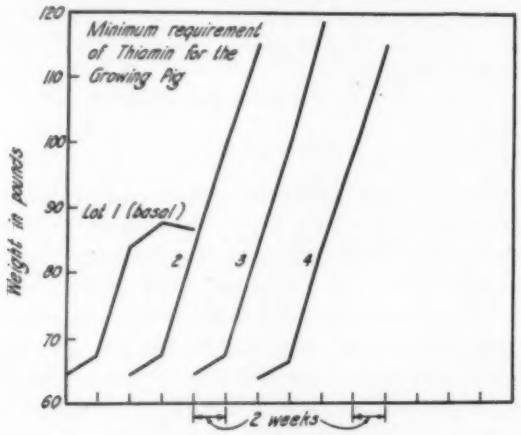

Fig. 1 Growth rates of growing pigs fed varying amounts of thiamin chloride. The average initial age of the pigs was about 65 days.

each week. A week's supply of each ration was mixed on the day the pigs were weighed and it was stored in paper lined, covered cans in a cool place and fed twice daily.

RESULTS AND DISCUSSION

Graphic representation of the results is shown in figure 1. All the pigs grew slowly the first week of the experiment. This was probably due to a change from a barley supplemented normal herd ration to a purified sugar supplemented diet. At the end of the fourth week those on the basal diet (without thiamin) began to exhibit a decline in their rate of

gain and their appetites were poor. At the end of the eighth week they were losing weight. To see if this was caused by a lack of thiamin, 1 mg. of thiamin was added to the diet of this group per 100 pounds of pig daily with the result that these animals gained 9 pounds each during the first week following the addition of this factor.

The rates of gain of the pigs in groups 2, 3 and 4 are very similar. The pigs in group 3 gained slightly faster than those in lots 2 and 4. There was one pig in this group which gained considerably faster than others in the experiment. One pig in each group was killed at the end of the eighth week of the experiment and a sample of the ham muscle of each was assayed for thiamin storage as a further check. From the growth rates and under the conditions of this experiment (fig. 1) it appears that the minimum requirement of thiamin for the growing pig is about 1 mg. per 100 pounds of pig daily. The results of the muscle assay (to be published in another paper) substantiate this assumption.

SUMMARY

Using a purified diet and with growth rate and muscle assay as the criteria, the minimum requirement of thiamin chloride hydrochloride appears to be about 1 mg. for 100 pounds of growing pig daily.

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THE CALCIUM AND PHOSPHORUS CONTENT OF CERTAIN VEGETABLES GROWN UNDER KNOWN CONDITIONS OF FERTILIZATION [1]

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(Received for publication May 8, 1940)

Much experimental work has been done regarding the amounts of fertilizer required by plants and the effect of these upon plant growth. Nearly all of the data concern the yield and quantity of the crop produced, rather than the effects upon quality and mineral content. Smith ('35) stresses the desirability of selecting for commercial purposes those strains of vegetables richest in minerals, realizing at the same time, that there may be considerable variation in composition of the same variety grown in different localities. Bishop ('34) has found that soils low in calcium content yield vegetables poor in calcium, and further that the addition of superphosphate increases the quantity of calcium only slightly. The Alabama soil from which Bishop obtained her vegetables was markedly different from the soil at Geneva. A résumé of pertinent literature to that date appears in her paper. More recent data related to this general subject are present in papers by Davidson and LeClerc ('36), Carolus ('38) and Willard and Smith ('38).

[1] The authors wish to acknowledge the assistance and cooperation of Profs. C. B. Sayre and W. D. Enzie of the New York State Agricultural Experiment Station at Geneva, New York.

[2] The data in this paper are taken from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Vital Economics of the University of Rochester, June, 1936.

Examples have been given by Hissink ('30) to show that while the proportions of mineral constituents in plants are dependent primarily on the plant itself, they are also somewhat dependent on minerals already in the soil or added as fertilizers. Coleman and Ruprecht ('35) have concluded that so-called complete fertilizers, when used in amounts for optimum crop production, exert little influence on the composition of crops. When heavy applications of fertilizer were used, effects of soil types on the composition of the crop were nullified.

MATERIALS AND METHODS

The object of this investigation has been to determine the effect of varying amounts of nitrogen, phosphorus and potassium, as supplied in commercial fertilizers, upon the moisture, ash, calcium and phosphorus content of certain vegetables and to correlate these data with certain others reported in the literature.

The vegetables examined in this study were corn (Golden Cross Bantam), green string beans (Stringless Refugee), beets (Detroit Dark Red), and cabbage (Glory of Enkuizen). They had been grown in the summer and harvested in early September at the New York State Agricultural Experiment Station at Geneva, New York. The land upon which the vegetables had been grown is part of a level plain located some 2 miles from the station proper, the soil being of the same type. Here plats, separated from one another by grass walks, had been laid out and each plat had received the same fertilizer treatment for a period of 8 years. Thus, soil conditions were uniform, the only variable being the fertilizer treatment. All crops were exposed to the same climatic conditions, so that for a series of observations made in one growing season, variations in temperature and rainfall may be ignored.

The string beans, corn and cabbage were harvested and brought to the laboratory on the same day. The beets, however, had been harvested a few days earlier and were removed from cold storage. Samples of each vegetable were taken from

plants situated in various sections of the plats to insure average and uniform quality. As soon as possible, the edible portion was prepared as for cooking, the beans and beets being washed free from dirt and wiped to remove adherent water. All samples were then passed through a meat grinder to secure a finely divided, fairly homogeneous mixture. Portions were removed at this stage and analyzed for moisture and ash. The

TABLE 1

Fertilizer used in amounts per acre

PLAT	AMOUNT USED	NH_3		P_2O_5		K_2O	
	pounds	*pounds*	*%*	*pounds*	*%*	*pounds*	*%*
A	600	24	4	96	16	24	4
B	300	12	4	48	16	12	4
C	1200	48	4	192	16	48	4
D	300	45	15	90	30	45	15
E	600	—	—	96	16	—	—
F	600	24	4	—	—	—	—
G*	600	24	4	96	16	24	4
H	None	—	—	—	—	—	—
I	600	24	4	96	16	—	—
J	600	—	—	96	16	24	4
K	600	24	4	—	—	24	4
L	600	48	8	96	16	24	4
M	600	24	4	192	32	24	4
N	600	24	4	96	16	48	8
X	150	6	4	24	16	24	4

* In addition this plat received 70 pounds of copper sulphate and 50 pounds of manganese sulphate per acre.

remainder of the ground material was dried and ashed carefully. Calcium and phosphorus in the ashes were determined in triplicate using procedures outlined in Methods of Analysis of the Association of Official Agricultural Chemists, third edition.

RESULTS

The data in table 1 show the amounts of fertilizer used per acre and the content of NH_3, P_2O_5 and K_2O in pounds and percentage. The size of each fertilized plat was $16 \times 76\frac{1}{2}$ feet. The area from which the crops were harvested on each plat

was $12 \times 72\frac{1}{2}$ feet which is exactly 1/50 acre. There was a space of 4 feet between plats to prevent fertilizers from adjacent plats being carried across during plowing and cultivation of the fields. Since the plats were considerably smaller than an acre, only the necessary proportion of fertilizer was used. In all cases the nitrogen in the fertilizer was derived half from ammonium sulphate and half from nitrate of soda. The phosphoric acid was in the form of superphosphate and potash in the form of the muriate. The superphosphate contained about 50% calcium sulphate, so that calcium was supplied to each plat receiving phosphorus fertilizer.

Plat H had received no artificial fertilizer treatment during the last 8 years, although cover crops were turned under each year. Plat X, or the check plat, received only a limited amount of fertilizer which was considered to be the minimum maintenance requirement. The best yields of all vegetables were obtained when the soil received the treatment given to plat A.[3] Plat G was given the same amount and kind of mixture as plat A, with the addition of copper and manganese sulphates.

The percentages of moisture, ash, calcium and phosphorus in the original samples are given in table 2. The data may be interpreted more readily by referring to table 3 during the discussion of results. The average moisture content of the corn, beans and beets was somewhat lower than that reported in the literature. The corn and beans seemed succulent and were being harvested for canning purposes. Since the beets had been in cold storage for a few days, it is possible that some water had been lost. Percentages of water content of all of the vegetables varied, fertilization having apparently little effect upon the corn and beans. The majority of fertilized beets contained less moisture than the lot raised on the check plat, the reverse being true for cabbage (table 3).

Beets were richest in ash being followed in order by corn, beans and cabbage. Considerable variation in ash content was observed among the samples of the four vegetables obtained

[3] Personal communication from Dr. C. B. Sayre, New York State Agricultural Experiment Station, Geneva, New York.

TABLE 2

Effect of fertilizers on content of certain minerals in vegetables

	BEETS					CABBAGE					CORN					BEANS				
Plat	H_2O	Ash	Ca	P	Ca:P	H_2O	Ash	Ca	P	Ca:P	H_2O	Ash	Ca	P	Ca:P	H_2O	Ash	Ca	P	Ca:P
	%	%	%	%		%	%	%	%		%	%	%	%		%	%	%	%	
A	82.1	1.14	0.028	0.035	0.80	93.5	0.61	0.052	0.029	1.80	69.9	0.79	0.0096	0.109	0.089	86.8	0.68	0.076	0.028	2.65
B	83.8	1.09	0.023	0.036	0.63	94.1	0.58	0.045	0.019	2.31	68.0	0.78	0.0084	0.114	0.074	86.3	0.79	0.080	0.036	2.24
C	85.6	1.17	0.024	0.047	0.50	93.5	0.59	0.049	0.024	2.06	69.8	0.73	0.0099	0.114	0.087	85.8	0.78	0.081	0.042	1.91
D	82.0	1.23	0.032	0.048	0.66	93.7	0.56	0.046	0.020	2.34	69.8	0.74	0.0052	0.112	0.047	86.5	0.85	0.080	0.040	2.00
E	81.1	1.15	0.031	0.035	0.88	93.7	0.61	0.046	0.027	1.69	66.8	0.76	0.0072	0.126	0.057	84.0	0.86	0.081	0.042	1.95
F	81.2	1.26	0.033	0.033	1.00	93.7	0.63	0.042	0.022	1.89	69.9	0.80	0.0117	0.097	0.120	85.7	0.71	0.077	0.035	2.20
G	82.9	1.16	0.017	0.044	0.38	94.9	0.58	0.043	0.022	2.00	68.1	0.88	0.0081	0.117	0.069	84.5	0.80	0.087	0.039	2.23
H	85.5	1.08	0.023	0.032	0.72	93.4	0.51	0.040	0.016	2.53	68.8	0.84	0.0127	0.116	0.109	84.6	0.75	0.082	0.031	2.64
I	76.2	1.32	0.032	0.059	0.53	92.8	0.66	0.046	0.025	1.85	70.3	0.85	0.0068	0.117	0.058	84.4	0.81	0.079	0.039	2.02
J	81.3	1.16	0.017	0.055	0.30	94.5	0.59	0.039	0.023	1.69	65.9	0.95	0.0137	0.138	0.099	85.4	0.75	0.085	0.035	2.44
K	81.7	1.11	0.027	0.036	0.75	93.3	0.63	0.041	0.022	1.84	67.7	0.83	0.0063	0.110	0.057	83.3	0.83	0.081	0.047	1.74
L	85.1	1.14	0.027	0.045	0.60	92.6	0.61	0.037	0.025	1.45	66.5	0.88	0.0066	0.132	0.050	83.2	0.79	0.078	0.036	2.14
M	85.6	1.13	0.022	0.049	0.45	94.4	0.55	0.043	0.027	1.60	70.9	0.88	0.0095	0.117	0.081	84.5	0.77	0.074	0.034	2.17
N	86.1	1.04	0.013	0.035	0.38	93.2	0.57	0.042	0.029	1.46	68.8	0.90	0.0077	0.138	0.056	83.7	0.85	0.068	0.053	1.29
X	83.4	1.04	0.028	0.024	1.13	93.4	0.62	0.038	0.024	1.59	69.5	0.92	0.0076	0.125	0.061	81.2	1.00	0.097	0.046	2.12
Av.	82.9	1.15	0.025	0.041	0.65	93.6	0.59	0.043	0.024	1.87	68.7	0.84	0.0087	0.119	0.074	84.7	0.80	0.080	0.039	2.12
Sherman			0.028	0.042	0.67			0.046	0.034	1.32			0.006	0.103	0.058			0.055	0.050	1.10
Bridges			0.024	0.037	0.65			0.045	0.026	1.73			0.006	0.103	0.058			0.050	0.051	1.02

from the various plats, but with the possible exception of corn, the fertilized plats yielded crops richer in ash than those grown on unfertilized soil.

There were marked variations in the calcium and phosphorus content of each of the vegetables examined. Our average percentages are different from those given by Sherman ('37), Bridges ('37) and others. The amount of calcium in corn and beans is considerably higher than that reported by Sherman ('37) while that in beets and cabbage is slightly lower. Since we removed the outer leaves of cabbage in preparing it for analysis, the percentage of calcium was probably

TABLE 3[1]

Summary—effect of fertilizers on ash, calcium and phosphorus

		MOISTURE	ASH EDIBLE PORTION	Ca		P	
				Edible portion	Ash	Edible portion	Ash
Corn samples compared with control—having	More	7	7	1	0	8	9
	Less	7	7	13	14	6	5
Bean samples compared with control—having	More	6	11	3	2	13	14
	Less	8	3	11	12	1	0
Beet samples compared with control—having	More	3	12	9	8	13	12
	Less	11	2	5	6	1	2
Cabbage samples compared with control—having	More	9	14	11	3	14	14
	Less	5	0	3	11	0	0

[1] A total of fifteen samples of each vegetable was analyzed. The number having more or less moisture, ash, calcium and phosphorus than the unfertilized controls are listed above.

reduced, for Cowell ('32) found the outer leaves contained much more calcium than the inner leaves. We found, as did Bishop ('34), that calcium and phosphorus tended to vary in an inverse manner, which caused marked differences in Ca : P ratios. A glance at table 3 reveals that fertilization reduced the calcium content of the edible portion of corn and beans only. Although the calcium content of the edible portion of cabbage is higher with than without fertilization, the reverse is true for the calcium content of the ash. This is due to the variation in the amount of ash present and its calcium content.

Thus, the cabbage raised on the control plat had the lowest ash content of any of the samples, but the percentage of calcium in this ash was higher than the average percentage for fertilized samples. For this reason the total amount of calcium present in the edible portion of cabbage grown on the check plat was low due not to a reduced calcium content of the ash but to the small amount of ash present in the sample. This accounts for the differences between the results for calcium and phosphorus in the edible portion and ash respectively as shown in table 3.

The data in tables 2 and 3 show clearly the effect of fertilizer in increasing the quantity of phosphorus in the ash and edible portion of vegetables. The larger applications of phosphate did not cause correspondingly greater contents of

TABLE 4[1]

Average percentage deviation of moisture, ash, calcium and phosphorus of all vegetables from those of control plat H

	CORN	BEANS	BEETS	CABBAGE
	%	%	%	%
Moisture	−0.19	+1.00	−3.28	+0.24
Ash	−0.92	+6.98	+6.34	+16.63
Calcium	−32.78	−7.31	−0.49	−6.27
Phosphorus	+4.16	+17.93	+20.26	+29.77

[1] See explanation of computation in text.

phosphorus in the vegetables. If the percentages of phosphorus found by us are compared with those reported by Sherman ('37), it is seen that with the exception of corn, our values are lower.

The average Ca : P ratios obtained with corn and beets are quite similar to those calculated from data collected by Sherman ('37), while those for beans and cabbage are considerably higher.

The figures presented in table 4 serve as a brief summary of the analytical findings on the ash itself. An example may be given to illustrate the method by which the data in this table are calculated. The percentage of a specific constituent obtained with the vegetable grown on the control plat is used

as a base. Variations from this are either plus or minus. Using values in table 2 for ash in corn, the percentage of ash in the control sample (plat H) is 0.844. In sample from plat A, this value is 0.794 or a difference of —0.050, or 5.92% of 0.844. After all of the values for each constituent had been so calculated, the plus and the minus percentages were grouped and added, the difference obtained and divided by 14—the number of fertilized samples. How application of fertilizer has reduced the calcium and increased the phosphorus of the vegetables is readily seen. This procedure was followed in obtaining data for calcium and phosphorus also, and all values

TABLE 5

Effects of individual types of fertilization upon ash, calcium and phosphorus contents

TYPE OF FERTILIZER	BEETS			CABBAGE			CORN			BEANS		
	Ash	Ca	P	Ash	Ca	P	Ash	Ca	P	Ash	Ca	P
	%	%	%	%	%	%	%	%	%	%	%	%
NPK Aver. 9 samples	1.13	0.024	0.040	0.59	0.044	0.024	0.84	0.0081	0.120	0.81	0.080	0.039
NP	1.32	0.032	0.059	0.66	0.046	0.025	0.85	0.0068	0.117	0.81	0.079	0.039
NK	1.11	0.027	0.036	0.63	0.041	0.022	0.83	0.0063	0.110	0.83	0.081	0.047
N	1.26	0.033	0.033	0.63	0.042	0.022	0.80	0.0117	0.097	0.71	0.077	0.035
PK	1.16	0.017	0.055	0.59	0.039	0.023	0.95	0.0137	0.138	0.75	0.085	0.035
P	1.15	0.031	0.035	0.61	0.046	0.027	0.76	0.0072	0.126	0.86	0.081	0.042
Cont.	1.08	0.023	0.032	0.51	0.040	0.016	0.84	0.0127	0.116	0.75	0.082	0.031

are based upon the percentage composition of ash and not upon edible portion of the vegetable.

If the effects of different types of fertilizers are considered separately, one finds that nine plats received additions of nitrogen, phosphorus and potassium, whereas one each received nitrogen and phosphorus, nitrogen and potassium, nitrogen, phosphorus and potassium, and phosphorus only. The validity of making comparisons between results from single plats with a single type of fertilization and the average result from a series of plats receiving similar fertilizers but in varying amounts may be questioned. We have, however,

grouped the data on this basis, but the findings are not clear cut (table 5). In the first place there is no consistent effect of any one type or kind of fertilization upon ash content. The calcium content of the edible portion of beets and cabbage is higher in the absence of potassium than with it, but the opposite is true for corn and beans. It would seem in certain instances that a base other than potassium may have replaced calcium in some crops raised on fertilized plats. The phosphorus content of the edible portion of beets, cabbage and corn is higher when phosphorus is used than when it is not, but this is not true for beans.

DISCUSSION

Hall ('10), Miller ('31), Sayre et al. ('31) and others have reported that plants utilize less calcium in the presence of abundant available potassium than in its absence. Little attention has been paid to this effect of fertilization on the calcium content of foodstuffs, and with the widespread use of fertilizers in crop production, it seems probable that the amount of calcium may be reduced considerably. Such considerations are of importance in national nutrition. Peterson, Elvehjem and Jamison ('25) determined the calcium and phosphorus present in samples of cabbage raised in several states, the variation in calcium being 0.029 to 0.056% of the edible portion and in phosphorus 0.017 to 0.057%. Our results are well within these ranges. This emphasizes the need of actual analyses of foods in studies of mineral metabolism.

The effect of phosphorus upon the calcium content of plants is a moot question. Newton ('28) believed that plants grown with liberal amounts of phosphate contained an increased amount of calcium, while Bishop ('34) found only a very slight increase in the calcium content of vegetables when superphosphate had been added to the soil. Our data confirm those of Bishop. Several workers, among them Hartwell ('13), Newton ('28), Clemm ('30) and Mitchell ('31) have shown that the use of phosphatic fertilizers leads to an increased

phosphorus content of the plant. This is clearly illustrated by the results in tables 2 and 3 of this paper.

In computing mineral intake, it is important that the food ingested be analyzed, since ash constituents are frequently lost during the cooking process. Further, in studies of mineral metabolism standard tables of analysis should not be relied upon in compiling mineral intake since marked variations occur from crop to crop and variety to variety. Likewise soil, temperature and other climatic conditions influence the mineral composition of plants, emphasizing again the need of chemical analysis to insure proper interpretation of results.

SUMMARY

A study has been made of the effect of various fertilizers upon the moisture, ash, calcium and phosphorus content of sweet corn, string beans, beets and cabbage grown on the same type of soil during the same season.

Corn and beans grown on fertilized plats contain less calcium than those grown on an unfertilized plat while all vegetables examined contained more phosphorus when given fertilizer treatment. We recognize the fact that this conclusion must be limited to apply to this particular soil and the fertilizers used. It is suggested that calcium may be replaced by another base, possibly potassium, supplied by the fertilizer.

Since the calcium and phosphorus contents of the vegetables vary widely and frequently in opposite directions, Ca : P ratios fluctuate between rather wide limits.

The importance of chemical analyses of food in metabolism studies is stressed because foodstuffs grown on different soil types under varying conditions of fertilization, culture, and climatic conditions may exhibit marked differences in composition.

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THE METABOLISM OF CITRIC ACID BY INFANTS[1]

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It has long been known that the salts of the organic acids occurring in fruits and vegetables are, with few exceptions, metabolized to alkaline end-products in the body. The extensive literature on this point has been reviewed by Smith and Orten ('37). Furthermore, the observation has been made that, like natural fruit juices, the ingestion of sodium citrate results in an increased pH of the urine and an augmented excretion of citric acid whereas an equivalent amount of free citric acid has little obvious influence on the composition of the urine (Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34). It has been shown that the alkalinization produced by sodium bicarbonate and by sodium acetate likewise increases the citric acid in the urine from which evidence the conclusion has been reached that the organism has the ability to synthesize citric acid (Schuck, '34a,b; Sherman, Mendel and Smith, '36a).

The distinct metabolism of free citric acid has been emphasized by the almost complete disappearance (insofar as examination of the urine is concerned) of this compound in adult human subjects (Östberg, '31; Kuyper and Mattill, '33; Schuck, '34b), in the dog (Sherman, Mendel and Smith, '36b), in the pig (Woods, '27; v. Fürth, Minnebeck and Edel, '34)

[1] A preliminary report was presented before the American Institute of Nutrition, New Orleans, March, 1940.

[2] Chas. Pfizer and Co. Research Fellow in Physiological Chemistry, 1938–39.

and in the rat (Kuether, Meyer and Smith, '40) after administration by mouth. Gonce and Templeton ('30) examined the urine of four children ranging from 7 to 12 years of age before and after giving 4 gm. of "dehydrated" citric acid per 15 pounds body weight. The results were irregular but in one subject the ingestion of the free acid resulted in a pronounced drop in the concentration of citric acid in the urine. In a study of the factors influencing the retention of calcium, phosphorus and nitrogen of infants who were fed whole milk, Jeans and co-workers ('36) made a comparison of the metabolism of the food citrate on the one hand, and of added citric acid, on the other. With four infants ranging in age from 7 weeks to 26 weeks, 0.088 to 0.206 gm. of citric acid were excreted in the urine when 1.47 to 2.72 gm. per day of food citrate were consumed; when, after adding free citric acid as a curdling agent to the milk, the citric acid intake was increased from 3.16 to 5.02 gm., the output remained at 0.097 to 0.213 gm. These data indicate complete destruction of citric acid when fed as such to this age group.

In their study of the metabolism of orally administered citric acid in the dog, Sherman, Mendel and Smith ('36b) found no increase in fecal citric acid when from 0.5 to 2.0 gm. of the free acid per kilo body weight was given. Langecker ('33) detected no citric acid in the feces of rabbits after feeding either sodium citrate or citric acid. The studies of Kuether, Meyer and Smith ('40) show that the feces of rats contain small amounts of citric acid and that the administration of free citric acid has little, if any, influence on the quantity lost by way of the intestine.

That considerable amounts of citric acid appear in the feces of human subjects was demonstrated by Smith, Bauguess and Barnes ('39) in a study of eleven infants from 3 to 14 months old; they reported analyses showing that from 1.0 to 7.8% of the citrate ingested by this group appeared in the feces. The present study is an extension of their work; it deals with the comparison of the metabolism of food citrate and of free citric acid as determined by the balance method.

EXPERIMENTAL PART

Methods

The subjects of this study were eight infants from 4 to 12 months of age with varying degrees of mild clinical rickets but without other abnormalities. They were given as much as they would voluntarily consume of a formula made up of dried milk, barley flour and *B*-lactose and also canned cereal, soup and apricot-apple sauce. Accurate account of food consumption was kept at all times. During the collection periods (3 days in length and marked by carmine) the babies were maintained on metabolism frames which permitted a complete separation of urine from feces and accurate quantitative collection of both. The data discussed herein are derived from one period during which free citric acid (365 mg./kg. body weight per day), was given by gavage in divided doses and one or two basal periods without added citric acid, for each subject.

The urine was collected in hydrochloric acid and the feces were digested with concentrated hydrochloric acid to a uniform suspension. For the determination of citric acid in the food, feces and urine, the method of Pucher, Sherman and Vickery ('36) was used, the final measurement being made photoelectrically, using a color filter having a maximum transmission at 4250 Å. Approximately 1 gm. samples of the separate food materials were thoroughly stirred with acid (70 cc. water and 3 cc. concentrated sulfuric acid) and extracted on a hot plate; after making to volume and filtering, the determination was made directly on the filtrate in most cases. The reconstituted dried milk was treated with trichloracetic acid and the determination made on the protein-free filtrate. The analyses for the citrate content of the food materials appear in table 1. Inasmuch as it was shown that all of the citric acid of the digested fecal suspension was in the fluid part, samples for analysis were measured directly with a pipette.

DISCUSSION OF RESULTS

It is obvious from the analyses of the food materials shown in table 1 that all of the constituents of the diet contained citric acid but that the dried milk and the canned fruit mixture were particularly rich in this respect. In the course of the experiment the food of the individual infants contributed from 1.091 to 2.502 gm. citric acid per day (see table 2).

In the report of Smith, Bauguess and Barnes ('39) attention was called to the not inconsiderable elimination of citric

Table I.
Citric Acid Content of Diet Constituents

Material	Citric Acid
	Per Cent
Barley Flour	0.047
* Milk Powder	1.400-1.625
B-Lactose	0.000-0.006
** Cereal	0.132
** Soup	0.078
** Apricot and Apple Sauce	0.638

* Klim
** Gerber's

acid in the feces although it was pointed out that the larger part of the excretion was through the urine. In the present study it is shown again that the feces always contain citric acid in varying proportions of the intake but ordinarily less than that appearing in the urine. The source of this citric acid may be the food, digestive or other body fluids entering the intestine or the activity of the intestinal bacteria. Incubation of known amounts of citric acid with infant feces for 44 hours produced, in our experience, neither destruction nor formation of citric acid. This observation agrees with that of Langecker ('34) who found that the bacteria and enzymes

in the intestinal tract of the rabbit do not destroy citric acid. In another study (Kuether, Meyer and Smith, '40) it has been shown that incubation of the intestinal contents of the rat with free citric acid for 44 hours results in no detectable destruction or formation of citric acid. In subjects ER, TA, WF and MC the large losses of citric acid in the feces during the period of free citric acid administration are correlated with diarrhea, probably induced by the citric acid itself. The food and possibly the intestinal secretions, thus appear to be responsible for the presence of citric acid in the feces. It is worthy of comment that, in the absence of diarrhea, fecal citric

Table II. CITRIC ACID BALANCE

Subject	Body Wgt.	Citric Acid Intake per 24 hrs			Citric Acid Excretion per 24 hrs			Excretion of Citric Acid in Per Cent of Total Intake			Citric Acid Absorbed in Per Cent of Total Intake	Urine Absorbed / 100	Urine Intake / 100
		Food	Extra	Total	Urine	Feces	Total	Urine	Feces	Total			
	KG.	GM.	GM.	GM.	GM.	GM.	GM.						
E.R. ♂	6.84	1.694		1.694	.169	.126	.294	9.9	7.4	17.3	82.6	10.7	9.9
	5.93	1.174	2.00	3.174	.093	.416	.511	3.0	13.1	16.1	87.0	3.7	3.1
A.K. ♂	8.33	1.909		1.909	.141	.048	.229	8.4	2.4	12.0	96.4	8.7	8.4
	9.76	2.302		2.302	.165	.079	.244	6.7	3.2	9.9	96.8	6.9	6.6
	8.19	1.091	3.00	4.091	.094	.032	.126	2.3	0.8	3.1	99.0	2.3	2.6
E.F. ♀	8.25	2.077		2.077	.160	.271	.431	7.7	13.0	20.7	87.0	8.8	7.7
	6.45	1.525		1.525	.128	.093	.221	8.7	7.0	16.7	93.0	10.4	9.4
	7.81	1.993	2.70	4.693	.062	.086	.148	1.3	1.8	3.1	98.0	1.3	3.1
T.A. ♂	10.34	2.464		2.464	.237	.073	.310	9.6	3.0	12.6	97.0	9.9	9.6
	9.24	2.140	3.30	5.440	.101	.630	.731	1.8	11.9	13.7	98.0	2.1	4.7
J.A. ♂	8.49	2.007		2.007	.230	.046	.296	11.5	2.3	15.0	96.5	11.9	11.4
	7.23	1.654	2.58	4.234	.160	.080	.240	3.8	1.9	5.7	98.0	3.9	9.7
R.P. ♂	7.03	1.328		1.328	.087	.067	.154	6.6	5.0	11.6	95.0	7.0	6.6
	6.05	1.786		1.786	.132	.173	.305	7.4	9.7	17.1	90.3	8.2	7.4
	6.87	1.672	2.58	4.252	.042	.340	.382	0.9	8.0	8.9	92.0	1.0	2.5
W.F. ♂	9.45	2.061		2.061	.248	.060	.308	12.9	1.9	14.8	98.1	13.1	12.9
	10.37	2.397		2.397	.231	.073	.294	10.0	3.3	13.3	96.7	10.3	10.0
	9.30	1.954	3.18	5.134	.072	1.000	1.072	1.4	19.5	20.9	80.0	1.8	3.7
M.C. ♂	8.85	1.760		1.760	.162	.059	.220	9.2	3.3	12.5	94.7	9.5	9.2
	7.48	1.628	2.78	4.408	.044	1.044	1.130	1.1	24.1	25.4	74.0	2.0	3.9

acid represents a lower proportion of ingested citric acid in the periods when the free acid is fed than in the control periods.

The above observations permit the conclusion that the citric acid which disappears from the intestinal tract is absorbed. From table 2 it can be seen that the proportion of ingested citric acid which is absorbed is high but is decreased in those instances where diarrhea was present. A different picture is presented by the data for citric acid excretion in the urine; here both absolutely and in relation to the quantity ingested, the values for the concentration of citric acid are strikingly

lower during the period when free citric acid was fed than in the control periods when the citric acid was obtained only from the food. A comparison of the proportion of ingested citric acid which was absorbed with the proportion appearing in the urine, provides an index of the completeness of metabolism of this substance. The thirteenth column in table 2 indicates that within the control periods and within the free citric acid-feeding periods, the extent of metabolism of the absorbed citric acid is uniform for the various subjects. However, when one compares the extent of metabolism of the free acid period with that of the control periods, there is a striking difference; the proportion of the absorbed citric acid escaping destruction averaged 9.6 (6.9 to 13.1) % in the control periods and 2.3 (1.0 to 3.9) % in the free acid periods. In the light of the failure to store citric acid in the body under ordinary conditions (Smith and Orten, '37) these data strongly suggest the interpretation that ingested free citric acid is either largely destroyed in the body or transformed to a non-citrate type of compound (see MacKay, Carne and Wick, '40).

A comparison of the amount of citrate ingested in the food with the concentration of urinary citric acid may be taken as an index of the influence of the diet on endogenous citric acid production inasmuch as both the salts of the organic acids occurring in foods and the major foodstuffs themselves, are known to affect citric acid synthesis. Such a comparison, shown in table 2, indicates that in seven of the eight subjects, the ingestion of free citric acid decreases the ratio of urinary to food citric acid, which fact may be interpreted to indicate that not only is the free citric acid metabolized but that, under its influence, the basal production of citric acid as affected by the diet, is inhibited. A similar comparison of the data from like experiments with the rat (Kuether, Meyer and Smith, '40), shows that, whereas the administered free citric acid completely disappears with this species it does not interfere to the same extent with the formation of urinary citric acid as influenced by the diet. The synthesis of citric acid, which can be demonstrated in the rat (Smith and Meyer, '39, Kuether,

Meyer and Smith, '40) is not so striking in the human subjects herein described.

Experimental data are available showing that under certain conditions, the excretion of citric acid by the kidney is suppressed. Thus the administration of hydrochloric acid or of potentially acid substances like calcium chloride and ammonium chloride, is followed by a decrease in citrate concentration in the urine (Östberg, '31; Kuyper and Mattill, '33; Boothby and Adams, '34). Again, Smith and Meyer ('39) have demonstrated that in the rat, the production of endogenous citric acid is greatly diminished when the animal is maintained on a high-protein diet. In the present investigation the failure to increase urinary citric acid apparently is not due to its effect on the acid-base equilibrium for it has been shown by Schuck ('34b) that ingestion of the free acid produces little if any variation of the pH of adult human urine and the same observation has been made in the rat (Kuether, Meyer and Smith, '40).

The present study shows again that the metabolism of free citric acid is not the same as that of food citrate or sodium citrate insofar as can be judged by the excretion of citric acid in the urine. That the metabolism of the free acid exerts a marked effect on that of food citrate is demonstrated by the data. From these balance experiments one can conclude that the fate of food citrates and of free citric acid in the infant is like that in adults and in the other mammals which have thus far been studied.

SUMMARY

Based on the data derived from a series of balance studies with infants, it has been shown that the oral administration of free citric acid results in a marked decrease in the proportion of the ingested citric acid and food citrate appearing in the urine, as compared with that of the control period in which the basal diet is the only source of citric acid. Ordinarily, the feces contain a small amount of citric acid; the citric acid of intestinal contents is uninfluenced by the addition of free citric acid.

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THE UTILIZATION OF CALCIUM IN CARROTS, LETTUCE AND STRING BEANS IN COMPARISON WITH THE CALCIUM IN MILK [1]

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Although it is generally known that the value of a food as a source of calcium in the diet depends upon the results of biological as well as chemical tests, foods are nevertheless evaluated largely on the basis of their contents of calcium. This situation is the result of the incompleteness of available information on the extent to which the calcium of different food materials is utilized in the animal body, and particularly in the human body.

With reference to carrots, the available information has been interpreted in support of quite discordant conclusions. The experiments of McClugage and Mendel ('18) on two dogs have been interpreted to mean that the addition of carrots and spinach to the diet "does not always yield a pronounced advantage to the calcium metabolism. It would, therefore, presumably be an unsafe procedure to use vegetables extensively as a dietary substitute for milk in the nutrition of children." Sherman and Hawley ('22) in experiments on children, concluded that "milk is much superior to vegetables as a source of calcium for the growing child." The vegetables compared with milk were a mixture of carrots, spinach and celery. Edelstein ('32) has also interpreted his metabolism data on children as indicating a decreased retention of

[1] This investigation was aided by the donation of funds by the American Dry Milk Institute to the University of Illinois.

calcium following the addition of carrots or spinach to the diet, consequent upon an impaired intestinal utilization. On the other hand, Rose's ('20) observations on women present no clear distinction between milk and carrots with respect to their values in furnishing calcium to cover the requirement of the adult organism.

These diverse conclusions regarding the value of carrots as a source of dietary calcium seem hardly to be justified from a critical study of the supporting data. The conclusion of McClugage and Mendel was based upon measures of the calcium utilization in the test foods obtained by expressing the negative calcium balances, secured in all experimental periods, as percentages of the calcium intakes. The greater these percentages, the poorer the utilization of dietary calcium was presumed to be. But such a calculation neglects the endogenous loss of calcium from the body. In making a correction for this body loss of calcium, it may be assumed, in conformance with recent evidence, of which the report of Fairbanks and Mitchell ('38) may be cited as an example, that the calcium of spinach is totally unutilizable in the animal body. This being true, the negative calcium balances of the dogs on the spinach diets would be equal to the endogenous calcium losses. These losses amount to 17.6 mg. of calcium per kilogram of body weight for dog A, and 21.6 mg. for dog B, values approximating the average of 18.3 mg. that may be computed from a number of experiments on dogs receiving low-calcium diets (Mitchell and Curzon, '39; see table 2). The differences between the negative calcium balances of the spinach periods and those obtained with the other test foods would measure the replacement of the endogenous calcium losses by the respective dietary forms of calcium. These replacements of calcium expressed as percents of the respective intakes of calcium would measure the utilization of the latter in the adult dog. For milk the percentage utilizations are 52 and 40 for dogs A and B, respectively, and for carrots, 24 and 29. These corrected values still indicate a marked superiority of milk calcium over carrot calcium, but the latter is distinctly

of value in the calcium metabolism of the body, being about 60% as valuable as milk calcium.

The poor showing of a mixture of vegetables including carrots in the calcium economy of the children in the experiments of Sherman and Hawley may be accounted for, to a large extent, by the inclusion of spinach in the mixture. This vegetable possesses the distinction, not only of containing calcium in an unutilizable form, but of probably impairing the utilization of the calcium of foods with which it is fed, because of its excessive content of oxalic acid.

The conclusions of Edelstein do not discriminate between the results with carrots and those with spinach. The addition of the latter food to the basal milk diet almost always induced a marked decrease in calcium retention, while the addition of carrots in two experiments led to discordant results, i.e., a marked decrease in calcium balance in one case, but a slight improvement in balance in the other. Such results justify no definite conclusion.

The experiments of Rose, involving intakes of calcium sufficient in all but one period (a carrot period) to induce slight retentions of calcium, may be unsuited to the detection of small differences between milk and carrots with respect to the dietetic value of their calcium contents. If the adult subjects of these experiments were saturated with respect to calcium, the consumption of calcium in amounts above those required for equilibrium would lead to a wastage of the excess intakes without regard to their inherent biological values. Under these conditions, differences in biological value would be obscured. It seems to be true that, in experiments on adult subjects, the relative values of different sources of calcium can be measured with assurance only if the subjects are in negative balance.

The only experiment concerned with the value of lettuce as a source of dietary calcium is the experiment of Mallon, Johnson and Darby ('33). The results secured were interpreted to mean that the calcium of lettuce was superior in its utilization by healthy women to that of pasteurized whole milk

fed at an approximately equal level of calcium. The foods tested accounted for 93 and 88%, respectively, of the total dietary calcium. But here, also, the calcium intakes were such that positive balances were secured in all periods. The fact that greater positive balances were secured in the first periods with each subject, when the lettuce diets were being consumed, may have been a result of an immediately prior period of inadequate calcium nutrition, rather than of a more utilizable calcium supply in the diet. This experiment again illustrates the difficulty of interpreting calcium metabolism studies on adult subjects in which more dietary calcium is provided than is needed for equilibrium.

The purposes of the experiments to be reported below were to measure the relative value in the nutrition of the growing animal of the calcium in milk and in carrots, lettuce and green string beans, and to determine whether cooking with steam and commercial canning processes modified the nutritive value of the calcium of vegetables. Since in these comparisons, and in the future ones that we plan to undertake, it is more convenient to use commercial skim milk powder [2] than fresh skim milk or fresh whole milk, a comparison of the value as sources of calcium of liquid skim milk and of the commercial powder was also made. This comparison would indicate whether commercial desiccation impairs the value of milk as a source of dietary calcium.

METHODS

The various experiments reported in this paper differed in many minor details of procedure, the nature of which it does not seem profitable to describe, but they were all carried out according to the following general plan.

The rats were taken for experiment at initial weights of about 30 to 50 gm. They were paired, or divided into groups of three (trios), on the basis of initial body weight (taken

[2] This product is now almost universally sold under the name "dry milk solids not over 1.5 pct. fat", and in the discussion below it will be referred to as "dry milk solids."

after a 24 hour fast), sex, and litter membership. Litter mate controls were sacrificed for analysis in order to estimate the initial content of calcium.

The basal diets were of two types. In the early experiments the protein, amounting to about 30% of the basal diet, was provided by dried, ether-extracted whole egg. These diets were fairly high in calcium, containing generally from 0.12 to 0.15%. With so much calcium in the basal diet, the proportion of the total calcium intake provided by the test supplements ranged from 40% up to only 80% depending upon the rate of gain, which determined the amount of supplement given. In the later experiments, the protein was provided by dried, ether-extracted beef round. These diets contained about 0.04% of calcium. The phosphorus contents of all basal diets ranged from 0.25 to 0.35%. The remainder of the basal diets contained in percentages, 10 of sucrose, 10 of lard, 10.5 of dried yeast, 1.5 of a fortified cod liver oil, 4.5 of a calcium- and phosphorus-free salt mixture, otherwise complete, and starch to make up the deficit.

The basal diet was fed in equal amounts to pair mates and trio mates, since the differences in calcium storage produced must be referable solely to the qualitative differences in calcium supply. In most contemporary publications in this field of investigation this precaution is not taken. The tacit assumption is made that the percentage of the calcium intake retained in the body is not influenced either by a variable intake of food or of calcium. This proposition has never been proved and seems inherently improbable. By equating the intake of food and of calcium for comparable animals, the uncertainties in interpreting the results of ad libitum feeding experiments are entirely avoided.

The test supplements were fed in addition to the basal diets in amounts to provide the same quantity of calcium to pair mates or trio mates, but less than that required for maximum calcification. Since maximal calcification involves the production of growth increments in body weight containing about 10 mg. (or somewhat more) of calcium per gram, the calcium

supplements were fed in amounts to provide only from 4 to 7 mg. of calcium per gram of gain, depending upon the concentration of calcium in the test food. With foods low in calcium, less calcium was fed per gram of body weight gain; but even so, enough of the test food had to be given to restrict seriously the intake of basal diet, which should provide most of the nutrients other than calcium. Hence, the basal diet was designed to be liberally provided with protein, minerals other than calcium and phosphorus, and vitamins.

The unequal amounts of supplements given to pair mates or trio mates to provide equal amounts of calcium, would tend to produce differences in body weight gains. Since unequal gains between (or among) otherwise comparable animals, even with equalized intakes of calcium, may modify the retention of calcium (Fairbanks and Mitchell, '36), the gains within pairs and trios were kept equal throughout the feeding period by giving sucrose in concentrated aqueous solution to the laggard rat.

The feeding was continued until the rats in each pair or trio had gained 90 to 100 gm. in body weight, requiring from 6 to 11 weeks, though ordinarily from 7 to 8 weeks. The rats were then sacrificed, the body length determined, the contents of the gastro-intestinal tract removed, and the carcass prepared for analysis for calcium, either by ashing all of it, or by freezing and grinding to a homogenous sample, weighed portions of which were ashed.

The test foods and basal rations in each feeding experiment were also analyzed for calcium. The well-known McCrudden method for the determination of calcium in biological materials was used, with slight modifications, in this experiment.

EXPERIMENTAL RESULTS

Liquid vs. dried milk. The first experiment involving a biological comparison of the calcium in unpasteurized liquid skim milk and in "dry milk solids" gave results indicating a superiority of the liquid over the dried milk. The difference was slight (5.2%), but statistically significant, the probability

of its being a fortuitous result being only 0.02. It was thought that the indicated effect of desiccation might possibly be due to a destruction of vitamin C in the milk during its desiccation. It seemed conceivable that, though the rat could demonstrably synthesize the vitamin, it might not be able to do so at a rate sufficient to secure the full advantageous effects on calcium metabolism. Hence, a series of experiments to test this possibility was carried out, and, as the results were all negative in

TABLE 1

Experiments concerned with the effect of desiccation on the utilization of the calcium of milk

EXPERIMENT NUMBER	GROUPS OF RATS AVERAGED	CALCIUM SOURCES COMPARED:	AVERAGE FINAL CONTENT OF CALCIUM IN CARCASS	ESTIMATED AVERAGE CALCIUM RETENTIONS	PROBABILITIES
			%	mg.	
1	8	"Dry milk solids" + vitamin C	0.850	1152	0.082
		"Dry milk solids"	0.785	1046	
2	8	Liquid skim milk + vitamin C	0.599	496	0.29
		"Dry milk solids" + vitamin C	0.609	508	
3	6	"Dry milk solids" + vitamin C	0.722	628	0.14
		"Dry milk solids"	0.689	592	
4	12	"Dry milk solids" + vitamin C	0.980	542	0.28
		"Dry milk solids"	0.991	549	
5	12	Liquid skim milk	0.747	555	0.21 [1]
		Dried skim milk	0.745	565	0.38 [2]
		Dried skim milk + vitamin C	0.743	558	0.15 [3]

[1] Liquid skim milk vs. "dry milk solids."
[2] Liquid skim milk vs. "dry milk solids" + vitamin C.
[3] "Dry milk solids" vs. "dry milk solids" + vitamin C.

significance, other experiments were performed to attack again the original problem of the effect of desiccation on the biological utilization of milk calcium. As the summary of the most significant results of these experiments (table 1) clearly shows, no considerable evidence was secured that the value of milk calcium for the growing rat was to any appreciable extent impaired by commercial desiccation of the milk. Also, no advantage in calcium utilization was conferred upon any of

the experimental rations by the addition of ascorbic acid to it at the rate of 1 to 3 mg. per rat daily. The last experiment (no. 5) was particularly convincing, not only because of its scope, twelve trios of rats being used as subjects, but because particular pains were taken to insure against the destruction of the ascorbic acid before consumption by the rat. These experiments confirm the negative results of Henry and Kon ('39).

After the five experiments of this series were completed and their results studied and analyzed, the data of the first experiment, the only one giving positive indications, were re-studied and re-checked. The anomalous result appeared to depend upon one very questionable analysis of a sample of liquid milk. Hence, the data of this experiment have not been reported.

The evidence here presented to the effect that the desiccation of liquid milk does not appreciably impair its value as a source of dietary calcium, receives some support from recent experiments in human nutrition. Kinsman and associates ('39), in prolonged experiments on eleven pre-school children, report an average utilization of the calcium in "dry milk solids" of 19% and an average percentage retention of the same magnitude. For an adult subject, Steggerda and Mitchell ('39) obtained a percentage utilization of the calcium of "dry milk solids" of 20. To be compared with these values is the average retention of 19% of the dietary calcium, of which fresh milk contributed 80%, reported by Pierce et al. ('40) for a group of ten pre-school children. These results indicate the existence of no appreciable difference in the availability of the calcium in fresh and in dried milk. The experiments of Kramer, Latzke and Shaw ('28) on the calcium and phosphorus metabolism of five children and seven adults receiving diets in which most of the calcium was provided by fresh, pasteurized, evaporated or dried milk, yielded quite contrary results, in that dried milk quite consistently proved a definitely poorer source of available calcium than fresh milk. Since the experiments involved a succession of short (6 day) metabolism periods, in which the different kinds of milk were tested, with

no intermediate adjustment periods, there is no assurance that the balances obtained are representative of the diets consumed.

"Dry milk solids" vs. carrots, fresh and steamed. The more significant average results of the three experiments involving a comparison of the calcium of "dry milk solids" with the calcium of fresh and steam-cooked carrots are summarized in table 2.

In experiment 1, the basal diet contained dried extracted whole egg as a source of protein and was hence fairly high in calcium content. The test foods supplied only 56% of the total intake of calcium. The amounts of calcium retained, both in milligrams and in percent of the intake, were greater in all fourteen pairs for the rats receiving the milk powder supplement. There can thus be no question of the significance of the difference as indicating a better utilization of milk calcium than of the calcium in fresh carrots. The average percentage retentions were 83.0 for the milk diet and 71.2 for the carrot diet. Thus the fresh carrot diet calcium was only 86% as well utilized as the milk diet calcium.

Experiment 2 consisted of a trio comparison of the calcium in milk solids and in raw and cooked carrots, eight trios of rats being used. In this experiment, because of the use of a basal diet containing a lower concentration of calcium, the test foods supplied 86% of the total calcium consumed. The average retention of calcium was considerably greater for the milk diet than for the carrot diets, being 467, 394, and 397 mg. for the milk, fresh carrot, and cooked carrot supplements, respectively, or 91.0, 76.7, and 77.3% of the consumed calcium. A statistical analysis by the method of Student ('08) reveals significant average differences between the milk diet and the fresh carrot diet ($M = 69.5$ mg., $s = 37.8$ mg., $P = 0.0046$) and between the milk diet and the cooked carrot diet ($M = 68.2$ mg., $s = 38.1$ mg., and $P = 0.0051$), but obviously an insignificant average difference between the fresh carrot diet and the cooked carrot diet. The values for P are the probabilities on a scale of one that the fortuitous factors alone would produce an average difference as large as, or larger than, that actually

TABLE 2

Average results of the experiments comparing the calcium of milk with that of carrots, lettuce and green string beans

	EXPERIMENT 1		EXPERIMENT 2			EXPERIMENT 3		EXPERIMENT 4		EXPERIMENT 5		
	Milk solids	Fresh carrots	Milk solids	Fresh carrots	Cooked carrots	Fresh carrots	Cooked carrots	Milk solids	Fresh lettuce	Milk solids	Fresh string beans	Canned string beans
Number of pairs or trios	14	14	8	8	8	6	6	10	10	12	12	12
Average daily gain, gm.	1.92	1.86	1.86	1.85	1.90	1.73	1.70	1.65	1.62	1.74	1.71	1.72
Attained body length, mm.	184.1	184.6	188.3	187.0	188.0	186.7	186.2	184	182	182	180	181
Calcium intake, mg.	713	713	522	522	522	481	481	500	500	419	419	423
Calcium intake in test food, %	56	56	86	86	86	85	85	80	80	90	90	90
Ash content of carcass, gm.	3.68	3.44	3.49	3.34	3.30	3.21	3.18	2.94	2.73	2.92	2.68	2.63
Ash content of carcass, %	2.78	2.64	2.37	2.32	2.25	2.34	2.30	2.33	2.20	2.57	2.41	2.34
Calcium content of carcass, mg.	952	872	836	754	762	764	744	665	584	687	588	579
Calcium content of carcass, %	0.719	0.671	0.585	0.543	0.536	0.552	0.543	0.526	0.470	0.605	0.528	0.516
Retained calcium, mg.	591	508	467	394	397	379	359	385	308	349	257	245
Percentage retention of calcium	83.0	71.2	91.0	76.7	77.3	78.5	74.3	76.8	61.4	83	61	60
Phosphorus content of carcass, mg.	—	—	—	—	—	—	—	—	—	569	528	512
Phosphorus content of carcass, %	—	—	—	—	—	—	—	—	—	0.501	0.474	0.456
Retained phosphorus, mg.	—	—	—	—	—	—	—	—	—	313	277	259

observed. The evidence seems clear in this experiment also that the calcium of milk is definitely better utilized by the growing rat than the calcium of fresh carrots, and, furthermore, that cooking carrots by steaming does not alter appreciably their value as a calcium food. If the calcium in the milk diet is given a value of 100, the values of the calcium of fresh carrots and cooked carrots are, respectively, 84 and 85.

In experiment 3, another comparison was made between fresh and cooked carrots, using six pairs of rats and rations such that 85% of the total calcium content was provided by the test foods. The mean difference (M) in stored calcium for the two diets was 20.0 mg., the standard deviation (s) was 24.8 mg., and the probability of a chance outcome is 0.065, too large to be neglected according to current biometrical practice. While the experiment thus offers some evidence for a better utilization of the calcium of fresh carrots than of the calcium of cooked carrots, it falls considerably short of a demonstration to that effect.

The fresh carrots used in the above experiments contained on the average 0.0325% of calcium and 0.0398% of phosphorus, and the dry skim milk, 1.200% calcium and 1.022% phosphorus.

"Dry milk solids" vs. lettuce. The relative value of the calcium in "dry milk solids" and in lettuce (containing 0.0140% calcium) was tested in experiment 4, using ten pairs of growing rats. Eighty percent of the calcium intake was contained in the test foods. The gains in body weight were slow, averaging only 1.63 gm. daily, and the percentage retentions were correspondingly, and perhaps consequently, low, averaging 76.8 for the milk diet and 61.4 for the lettuce diet. The mean difference in calcium retention was 77.5 mg., the standard deviation of differences was 31.2, and the probability of a fortuitous difference (Student, '25) was 0.00002, so very small that it may be neglected. As further evidence of the superiority of the milk supplement, the average body length of the milk rats was significantly ($P = 0.025$), if only slightly ($M = 1.4$ mm.), greater than that of the lettuce rats.

In this experiment the lettuce calcium was only 80% as well utilized as the milk calcium.

"Dry milk solids" vs. green string beans. The relative values in nutrition of the calcium in "dry milk solids" and in fresh and commercially canned green beans were determined in experiment 5, using twelve trios of rats. In this experiment, the phosphorus metabolism as well as the calcium metabolism was measured. As with the carrot experiment, the difference in calcium utilization was statistically significant between the milk solids and the raw vegetable ($P = 0.00001$), but was quite insignificant between the raw and the canned vegetable. The retention of phosphorus was also distinctly better on the milk diet than on the fresh string bean diet ($P = 0.0062$). In this experiment the test foods provided 90% of the total calcium consumed.

That the milk diet promoted a more rapid calcification of the bones than the vegetable diets, is also shown by the higher ratio of retained calcium to retained phosphorus, these ratios being 1.11 to 1 for the milk diet, 0.93 to 1 for the fresh bean diet and 0.95 to 1 for the canned bean diet. The calcium in the bean diets was only 73% as available to the growing rats as the calcium in milk solids.

DISCUSSION

The calcium of the vegetables tested in this study is quite definitely less available to the growing rat than the calcium of milk. Since considerable differences have not been found in the utilization by animals of calcium salts appreciably soluble in water (Bethke, Kennard and Kick, '29; Deobald, Elvehjem, Hart and Halpin, '36; Mitchell, Carroll, Hamilton, Garrigus and Hunt, '37) if adequate amounts of vitamin D are provided, and since a number of calcium salts, both organic and inorganic, have been shown to be as valuable in nutrition as the calcium compounds of milk (Stearns and Jeans, '34; Steggerda and Mitchell, '39; Pierce et al., '40), it would appear that the differences noted between milk and vegetables are the result of the presence in vegetables of constituents, possibly

carbohydrate in nature, that impair calcium absorption from the intestinal tract.

These constituents may act by rendering the calcium compounds in vegetables less accessible to the solvent action of the digestive juices, in much the same fashion as the indigestible carbohydrates impair the digestion of cereal and legume proteins (Mendel and Fine, '11a; '11b; '11c; '12). However, the fact that the cooking and commercial canning processes, that would be expected to rupture cellular membranes, do not improve the utilization of vegetable calcium, would militate against this explanation.

A second possibility is that the constituents of vegetables responsible for the impairment in calcium utilization act either by combining with the calcium to form insoluble compounds, as the oxalates of foods are known to do, or to modify unfavorably the reaction of the intestinal contents, or to increase intestinal motility. These factors must account for the so-called "associative effects" of foods in digestion, whereby one food will impair the digestibility of another. If this possibility is correct, then not only will the calcium of vegetables be less completely utilized than the calcium of milk, but the presence of vegetables in the diet will depress the absorption of the calcium compounds in foods with which they are fed, even in milk.

Evidence favoring the latter explanation may be found in the results of experiments 1 and 2 (table 2). In experiment 1, fresh carrots furnished only 56% of the calcium intake, the remainder being provided to a considerable extent by dried egg. In experiment 2, fresh carrots provided 86% of the calcium intake. Nevertheless, the percentage retention of calcium was approximately the same, 71.2 and 76.7, respectively, and in comparison with the calcium of the milk diets, the calcium of the carrot diets was of equal value, i.e., 86 and 84% as good, respectively. Apparently, the addition of the non-calcium constituents of carrots to the basal diet in experiment 1, impaired the utilization of the calcium of the carrots and of egg to approximately the same extent. Tso ('26) has re-

ported experimental observations indicating a very high utilization of the calcium of egg.

SUMMARY AND CONCLUSIONS

In a series of ten experiments on growing rats, involving equalization of calcium intakes and of gains in body weight among comparable animals, the calcium retention was determined by carcass analysis following a period of experimental feeding sufficiently long to permit gains in weight of 100 gm. The availability of the calcium of various vegetable foods and of unpasteurized liquid milk was compared in each case with that of the calcium of "dry milk solids" at levels of calcium intake insufficient to promote maximum calcification of the bones. The results obtained justify the following conclusions:

1. The commercial desiccation of milk does not appreciably impair the value of its calcium in the nutrition of growing animals.

2. The calcium of milk is definitely better utilized than the calcium of the vegetables tested. Under the conditions of these experiments, the calcium of fresh carrots, fresh lettuce and fresh green string beans was 85, 80, and 74%, respectively, as available as the calcium of milk.

3. The steam cooking of carrots and the commercial canning of green string beans do not modify appreciably the value of these vegetables as sources of dietary calcium.

4. The constituents of vegetables tend to depress the utilization of the calcium of other foods with which they are fed. The maximum extent of this effect may be determined by the extent to which the calcium of the vegetable, when fed as the sole source of calcium, is utilized in comparison with the calcium of milk.

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THE UTILIZATION OF THE CALCIUM OF DI-CALCIUM PHOSPHATE BY CHILDREN [1,2]

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ONE FIGURE

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INTRODUCTION

Recent studies carried out in these laboratories have yielded data which show clearly that man is remarkably uneconomical in his utilization of milk calcium. In the carefully controlled experiments of Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell ('39), the well-nourished subjects were able to use only one-fifth of the calcium eaten in this form. Such wastage with respect to a food which has long been considered superior as a conveyer of calcium would warrant further investigation of other sources which may, possibly, yield their calcium more readily. The question arises as to whether or not the pure calcium salts, which are frequently recommended by physicians as a supplement to the diet, are better than milk in this respect. Inasmuch as di-calcium phosphate gave good calcium retention in the babies studied by Stearns and Jeans ('34–'35), this salt was selected for the present study. If its calcium were unusually well utilized, this inorganic compound

[1] A preliminary report of some of these data was given before the American Public Health Association, Kansas City, October, 1938, and before the American Institute of Nutrition, New Orleans, March, 1940.

[2] Aided by a grant from the American Dry Milk Institute, Inc., Chicago.

[3] A portion of these data was presented by Miss Herta S. Breiter in partial fulfillment of the requirements for the degree of Master of Science at the University of Illinois, June, 1939.

would be a valuable adjunct to human dietaries in view of its relatively high calcium and phosphorus content, its palatability and its low cost.

EXPERIMENTAL

The subjects used in this experiment were six pre-school boys who were well adapted to the demands of the experiment since they had participated previously in a study of the utilization of milk calcium. For 9 weeks immediately preceding the present study, they had been fed calcium at a generous level (i.e., 900 mg. daily) in an attempt to saturate their body stores with this element. Throughout all these metabolism studies, totalling 54 weeks, the subjects were maintained on a constant regimen. This regimen was essentially the same as that described in a previous study with girls (Outhouse, Kinsman, Sheldon, Twomey, Smith and Mitchell, '39); however, an increase in the quantities of cereals and potatoes was necessary in order to satisfy the higher caloric needs of these boys who were heavier than the girls. The ages, heights and weights of the boys at the end of period VI and their gains in weight during the 12 months preceding the end of the study are given in table 1. The procedures for the collection of metabolic materials were identical with those of the previous studies; this was also true for the analytical techniques.

Inasmuch as the purpose of this study was the securing of a quantitative measure of the availability for growth purposes of the calcium of a specific compound, a procedure was used which would give a value that would be uninfluenced by the subject's maintenance requirement for calcium. This procedure, proposed by Mitchell in a previous study on milk calcium (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39), consists of determining calcium balances at two levels of intake, the difference in the levels being due to the calcium content of the substance under investigation. In computing percentage utilization, the difference between the calcium intakes at these two levels and the difference between calcium retentions were used. By considering only the incre-

ment in retention which was induced by the increment in intake, one can differentiate between the retention relating to the total intake and the retention resulting from the ingestion of the test substance. In this laboratory, the latter is interpreted as being representative of utilization.[4]

Obviously, this procedure necessitates that both levels of calcium intake be sufficiently generous to allow for positive balances (i.e., they must exceed the subject's maintenance requirement) but not for maximal storage of calcium. Since, in previous studies on these same, and other, children, a daily intake of 500 mg. did not result in maximal calcium retentions, this amount of calcium was chosen as the higher level in the present experiment; of this total intake, 126 mg. were fed in the form of $CaHPO_4$. The calcium salt was weighed in weekly allotments of exactly 3.8 gm. for each child; this amount was divided into seven approximately equal parts each of which was placed in a gelatin capsule. One capsule was given each morning at breakfast. This procedure was continued until balances for an additional week did not change appreciably the values which were obtained on averaging the calcium balances for consecutive preceding weeks; this time interval (i.e., 7 weeks) is designated as period VI. Subsequent to this period, calcium was fed at a lower level; this low level of intake was achieved by removing the supplement of $CaHPO_4$. The length of this period (period VII) varied for the different subjects; it ranged from 7 to 13 weeks. During the first few weeks, subjects Br and Jw retained such small quantities of calcium that it was deemed advisable to discard the data for those weeks and to continue the experiment for at least 6 weeks after the retentions had become more satisfactory and more constant. Subjects R and G withdrew from the experiment at the beginning of this period; therefore, it was neces-

[4] In this discussion use is made of the terms "per cent retention" and "per cent utilization"; they are not synonymous since per cent retention values will be lower than per cent utilization values due to endogenous losses. However, for children who apparently have little or no maintenance requirement, these terms are probably interchangeable.

sary, in computing percentage utilization, to use the values observed for these two boys during an earlier period (period II). Because the respective sources and intake levels of calcium for periods II and VII were identical, such a substitution seemed reasonable.

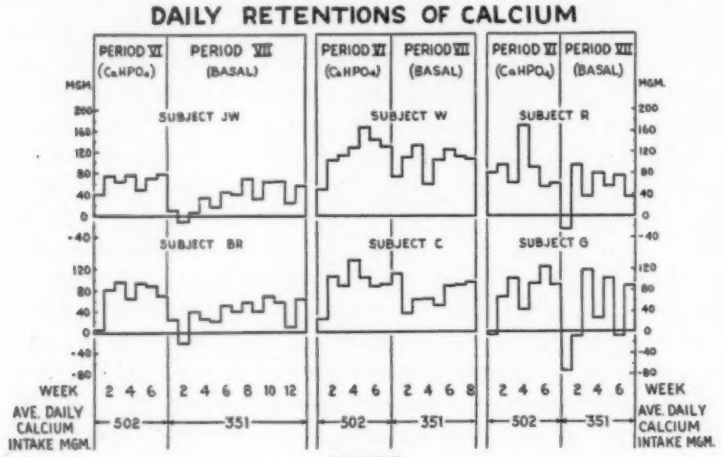

FIGURE I

RESULTS

The data pertaining to this study are recorded in figure 1 and in table 1. During the period in which the subjects received the basal diet, the daily calcium intakes ranged from 328 to 364 mg. with an average of 351 mg. For the individual subjects R, W, C, G, Br and Jw, these intakes were 328, 359, 364, 343, 357 and 353 mg., respectively. The corresponding average daily retentions were 63, 107, 69, 53, 48 and 45 mg., and averaged 64. When these retentions are expressed as a percentage of the intake, they average 18.3 with individual values of 19.2, 29.8, 19.0, 15.5, 13.5 and 12.8, respectively, for the subjects in the order listed above.

When di-calcium phosphate was fed, the calcium intakes averaged 502 mg. daily. Subjects R, W, C, G, Br and Jw re-

tained, as daily averages, 89, 131, 103, 87, 83 and 69 mg., respectively, on intakes of 483, 493, 512, 517, 512 and 492 mg. These retentions represent, respectively, 18.4, 26.6, 20.1, 16.8, 16.2 and 14.0% of the intakes; the average value for the group is 18.7%.

As stated in a preceding paragraph, the method adopted in this study for computing the percentage utilization of the calcium in $CaHPO_4$ makes use of the difference in the quantity

TABLE 1

Data on the utilization of the calcium of di-calcium phosphate

SUBJECTS		R	W	C	G	Br	Jw	Ave.
Age in years at end of period VI		3.5	4.5	5.6	6.1	6.5	6.5	
Weight in kg. at end of period VI		17.4	18.4	21.3	21.2	23.2	21.1	
Height in cm. at end of period VI		102	109	117	117	122	124	
Weight gain (ave. daily) in gm. during 12 mo. prior to end of period VII		7.2	10.2	6.4	5.4	4.4	5.8	
$CaHPO_4$ + basal diet (period VI)	Days on experiment [1]	42	42	42	42	42	42	
	Calcium intake (ave. daily) in mg.	483	493	512	517	512	492	502
	Calcium retention (ave. daily) in mg.	89	131	103	87	83	69	94
	Calcium retention, %	18.4	26.6	20.1	16.8	16.2	14.0	18.7
Basal diet (period VII)	Days on experiment [1]	42	49	49	42	56	70	
	Calcium intake (ave. daily) in mg.	328	359	364	343	357	353	351
	Calcium retention (ave. daily) in mg.	63	107	69	53	48	45	64
	Calcium retention, %	19.2	29.8	19.0	15.5	13.5	12.8	18.3
Utilization of calcium of $CaHPO_4$		16.8	17.9	23.0	19.5	22.6	17.3	19.5

[1] Exclusive of first week except for subjects Br and Jw who required 5 and 3 weeks, respectively, for adjustment to the low level of intake of period VII.

of calcium retained during the basal period and during the $CaHPO_4$ period and of the difference in the quantity of calcium ingested during the two periods. In the data presented in the table, it will be noted that the difference in the average calcium intakes of periods VI and VII was 151 mg. rather than 126 mg.—the amount of calcium furnished by the $CaHPO_4$. In other words, less food calcium, to the extent of 25 mg., as an average, was eaten during the basal period.

This unforeseen deviation from the experimental plan to have the difference in calcium intakes due solely to the $CaHPO_4$ supplement can be accounted for almost entirely by an increase in the consumption of bread by every child during the time he was ingesting $CaHPO_4$. Although the bread was made with water and contained only 0.033% calcium, it contributed a significant and variable amount of calcium to the diet since the children were allowed to eat it ad libitum as a means of satisfying individual caloric needs. Consequently, the difference in retention of calcium between periods VI and VII was not due entirely to the ingestion of $CaHPO_4$; approximately one-sixth of the difference in calcium intake must be attributed to the constituents of bread. On the basis of this mixture of calcium, 83% of which was derived from $CaHPO_4$, the following utilization values were obtained for subjects R, W, C, G, Br and Jw: 16.8, 17.9, 23.0, 19.5, 22.6 and 17.3%, respectively. These values average 19.5%.

DISCUSSION

The six pre-school boys who served as subjects in this study made poor use of the calcium of di-calcium phosphate; i.e., they retained only approximately 20% of the calcium of this salt when it was fed under conditions which were designed to demonstrate maximal calcium utilization. Such great wastage of the ingested calcium does not necessarily contraindicate the use of this inorganic salt as a source of calcium for children, inasmuch as equally great calcium wastage was observed in these same subjects during an earlier study in which milk supplied an almost comparable quantity of calcium (Kinsman et al., '39). The percentage utilization of milk calcium by subjects R, W, C, G, Br and Jw proved to be 17.8, 21.5, 20.0, 12.7, 19.1 and 27.5, respectively. Although each subject did not make use of exactly the same amount of calcium from both sources, the utilization values are of the same order of magnitude, and the averages for the two groups are almost identical —i.e., 19.5% and 19.8%. This finding—that there is no essential difference in the extent to which the calcium in these two

substances is utilized by children—agrees with the conclusions drawn by Stearns and Jeans ('34–'35) from their study of pre-school children. Jansen ('24) reached a similar conclusion in his study on an adult. These are the only experiments which have been found in the literature in which milk and di-calcium phosphate were compared.

However, other calcium salts have been fed to children of the same age-range as those of the present study. Stearns and Jeans ('34–'35) fed the calcium salts of di- and tri-phosphates, lactate, carbonate and gluconate, singly or in conjunction with milk, but neither percentage retention nor percentage utilization values can be computed from their data since they were reported as group averages. These investigators felt that there was no difference in the availability of the calcium of these salts with the exception of gluconate calcium; two 6-year-old children were in negative balance when they received calcium gluconate. (However, the children on the 7 to 12 age-group apparently were able to make just as good use of this salt as they did of milk or of $CaHPO_4$.) One of Grosser's ('20) subjects was a pre-school child—he was $2\frac{3}{4}$ years old; his utilization of the calcium of calcium lactate amounted to 24%. But, in view of the short duration of both of these experiments, little weight can be placed on the data.

That growing children, under certain conditions, might not exhibit an appreciable maintenance requirement for calcium was suggested in a previous publication (Kinsman et al., '39). This belief is based on two facts; namely, that in the eleven children studied (a) no consistent difference existed between the values for percentage retention and those for percentage utilization and (b) the values for percentage retention at different levels of intake, instead of increasing with increasing calcium intake, remained constant. The data of the present study carry the same implication. Although the average values for percentage retention during periods VI and VII are lower than the average value for percentage utilization of the calcium in $CaHPO_4$, this difference is not found consistently for the individual subjects. This inconsistency is obvious in the

following comparison of values for percentage retention and percentage utilization of 18.4 and 16.8, 26.6 and 17.9, 20.1 and 23.0, 16.8 and 19.5, 16.2 and 22.6 and of 14.0 and 17.3, respectively, during period VI for subjects R, W, C, G, Br and Jw. Moreover, the average values for percentage retention of the calcium ingested during period VI and VII are practically identical; i.e., they are 18.3 and 18.7 for the respective periods. Such an analysis of the data herein presented supports the contention that, at levels of intake which do not exceed the minimal requirement for calcium for maximal storage, growing children show little, if any, requirement for calcium for maintenance purposes. The practical inference arising from this belief is the previously expressed suggestion that, in formulating the child's dietary, insofar as calcium is concerned, only the requirement for growth purposes probably need be considered.

SUMMARY AND CONCLUSIONS

Six little boys, ranging in age from 3½ to 6½ years, were fed two levels of calcium, both of which were below the minimal requirement for maximal retention but obviously in excess of a possible maintenance requirement. The lower level contributed, as an average, 350 mg. of calcium daily, the higher one, 500 mg. daily; the difference was due, mainly, to the daily supplementation of the basal dietary with 543 mg. of di-calcium phosphate. The utilization of the calcium averaged 19.5%; individual values were 16.8, 17.9, 23.0, 19.5, 22.6 and 17.3%. These same children had previously served as subjects in a study of the availability of milk calcium and had been able to utilize only 19.8% of that calcium; therefore, it may be concluded that di-calcium phosphate is not superior to milk as a source of calcium for children and that, except for the person who is allergic to milk, there is little virtue in recommending the replacement of milk in the diet by di-calcium phosphate.

ACKNOWLEDGMENT

Grateful appreciation is herewith expressed for the cooperation extended by Mrs. Charlotte Fitzgerald, superintendent, and the members of the Board of Trustees of the Cunningham Children's Home, where this experiment was conducted, and by Miss Charlotte Beard and Miss Nellie Ratcliffe, dietitians. The authors also wish to acknowledge their indebtedness to Dr. H. H. Mitchell, of the Animal Husbandry Department, University of Illinois, for his continued interest in and support of these studies; his counsel has been invaluable.

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PECTIC ENZYMES
V. THE FATE OF PECTINS IN THE ANIMAL BODY [1]

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ONE FIGURE

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For several reasons the fate of pectins in the animal body is of interest. The pectins of various fruits and vegetables are a part of the regular diet and although they are present in small quantities it would be of importance to know whether they are utilized as certain other polysaccharides are. Pectin is also an important component of the apple diet often successfully employed to combat diarrhea. The usefulness of this treatment is generally recognized (Council on Foods, American Medical Association, '37) but its working mechanism has not yet been satisfactorily explained. No final answer to this question can be expected until the metabolic fate of the various components of apples is clear.

All investigators who have studied this question agree that pectin added to the diet of animals and human subjects cannot be recovered from the feces. Fürth and Engel ('31), Manville, Bradway and McMinis ('36) and others believe that pectin is a digestible carbohydrate, hydrolysed and utilized in the animal body, but there is no evidence supporting this assumption. Murer and Crandall ('40) suggest that pectin is not hydrolysed in the digestive tract of the dog.

[1] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 379, April 2, 1940. These investigations have been supported by the Harold H. Clapp, Inc. Grant. Presented at the Cincinnati meeting of the American Chemical Society, April, 1940.

An investigation of the working mechanism of the apple diet was begun at this Experiment Station some time ago and in connection with it various tests were conducted to obtain information on the fate of pectins in the animal body. The results obtained are presented herewith. A method for the determination of possible slight decomposition of pectins is also described with the hope that it will facilitate further investigations in this field.

METHODS AND MATERIALS

The powdered citrus pectin used was exhaustively extracted with cold 50% alcohol and contained 88.4% uronic acid (Lefèvre and Tollens, '07, expressed as polygalacturonic acid) and 10.28% CH_3O determined by saponification. It also contained 0.49% ash, and 1 gm. of the pectin gave 1.09 gm. of calcium pectate (Carré and Haynes, '22). The pectin was thus composed almost exclusively of polygalacturonic acid in which most of the carboxyl groups were esterified.

It may be noted that a preliminary precipitation of the pectins with acidified alcohol is usually desirable. The alcohol precipitate is dissolved in a small volume of hot water and this solution used for the determination of calcium pectate and also of the relative viscosity by an Ostwald viscosimeter at 30°C.

In these experiments it was necessary to use a method by which any possible small digestion could be determined on a portion of the pectin applied. It has been reported previously (Kertesz, '39) that upon the slightest decomposition of pectin, there is a great decrease in its viscosity. This causes the ratio $\frac{\text{calcium pectate}}{\text{relative viscosity}}$ to increase materially. The method is applicable only in cases of slight decomposition because upon extensive hydrolysis the calcium pectate precipitate disappears completely.

In all in vitro experiments 30°C. was used rather than body temperature because the degeneration of the viscous properties of pectin is slower at this temperature. There was also less likelihood of slow heat inactivation of the various en-

zymes at this temperature during prolonged digestion. Finally it was also desirable to have the reactions proceed at the same temperature at which the viscosity determinations were performed. In order to avoid hydrolysis or saponification of the pectin by the medium, all in vitro experiments were made at pH 3.4 and 6.4.

EXPERIMENTAL RESULTS

The observation that pectin added to the diet is digested in the body was substantiated by several experiments on dogs as well as on human subjects. In not a single case could any pectin be found in the feces, indicating that it was completely hydrolysed. To discover the agencies responsible for this hydrolysis various digestive secretions were examined for their effect on pectin.

Saliva collected from human subjects, dogs and cows had no effect on pectin at pH 3.4 and 6.4 during several weeks of incubation in the presence of toluol.

Jejunal juice was collected from an isolated auto-transplanted segment in a dog; about 12 ml. were obtained in 4 hours. It contained very active saccharase, amylase and peptidase. To test its possible pectolytic activity 0.5 ml. portions of it were digested with 5.0 ml. of a 1.5% pectin solution in the presence of toluol for several days. Tests performed at various times up to 3 days gave no indication of the slightest decomposition.

Attempts to digest pectin in the stomach and small intestine of the dog failed. The dog used in this experiment weighed 14 kg. and had an enterostomy in the lower end of the jejunum. Previous experiments on the same dog indicated that, of 48 gm. available carbohydrate in test meal ingested, only 15 to 20% could be recovered from the fistula. With the aid of a stomach tube 45 ml. of a 2.0% solution of pectin, followed by about 25 ml. of water, was introduced. The dog showed no signs of discomfort.

At intervals of 60, 90 and 160 minutes 36 ml., 41 ml. and 10 ml. liquid respectively were obtained. In the combined

samples the pectin was precipitated and washed with alcohol, dried at room temperature and dissolved in water; 98.8% of the pectin introduced was recovered. The relative viscosity of this solution when compared with that of the original solution of the same pectin content gave no indication of hydrolysis.

In order to determine whether pectin is digested in the human stomach 200 ml. of a 1.23% solution of pectin was ingested by an adult male. Several samples of 13 to 25 ml. were

TABLE 1

Digestion of pectin in the human stomach and by pectinase

NO.	SAMPLE	DURATION OF EXPERIMENT	PECTIN PURIFIED FROM THE STOMACH CONTENTS OR DIGEST			CALCIUM PECTATE / RELATIVE VISCOSITY OF REFERENCE SOLUTION OF SAME PECTIN CONTENT (2)	DIFFERENCE BETWEEN (1) AND (2)
			Calcium pectate	Relative viscosity (water = 1)	Calcium pectate / Relative viscosity (1)		
		minutes	%				
1	Recovered by stomach tube	45	0.230	2.11	0.109	0.102	+0.007
2	Same as no. 1	90	0.137	1.79	0.077	0.081	−0.004
3	Incubated with juice from stomach	1440	0.393	3.25	0.121	0.117	+0.004
4	Vomited	30	0.117	1.46	0.076	0.071	+0.005
5	Digested with 0.1% commercial pectinase	5	0.452	2.52	0.179	0.129	+0.050
6	Same as no. 5	15	0.428	2.01	0.213	0.129	+0.084

recovered by stomach tube at intervals up to 90 minutes. The person on whom the experiments were performed was accustomed to swallowing the stomach tube and not the slightest discomfort was indicated by him from this operation or from any effect of the pectin. The samples obtained were examined separately and the results are shown in table 1. This table also shows the results obtained in an experiment in which a 5 ml. sample of juice taken from the stomach of the same person was incubated with 10 ml. pectin solution for 24 hours.

All results indicate the absence of digestion. Experiments with pectin ingested by an adult and vomited after 30 minutes also gave negative results.

Tests were conducted to determine the possible digestion of pectin by trypsin, pepsin, rennet and pancreatic amylase. Analyses of the reaction mixtures after 12 days' incubation showed that the pectin was intact.

To study the effect of feces on pectin a suspension was made of human feces and the mixture was filtered through cloth to remove the larger particles. From this suspension (containing 6.31% solids) 50 ml. aliquots were mixed with 25 ml. of a 1.25% pectin solution and the mixture incubated at 30°C. At

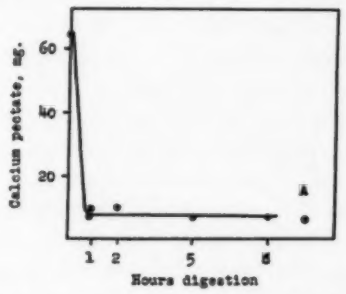

Fig. 1 Digestion of pectin with feces suspension. Sample "A" was digested with feces suspension plus 1% very active pectinase for 48 hours.

intervals up to 8 hours the digests were mixed with 300 ml. of 95% alcohol, the precipitate was dissolved in hot water, made up to volume and an aliquot representing one-fifth of the original mixture was used for the precipitation of calcium pectate. The results obtained are presented in figure 1. The pectin was rapidly digested by the feces suspension. Under these conditions a small quantity of material present in the feces suspension gave a precipitate which did not disappear after prolonged digestion with pectinase. The pectin was also decomposed by feces in the presence of toluol although the progress of the digestion was irregular and much slower in

this case. Canine feces gave results similar to those shown in figure 1.

DISCUSSION

Saliva and the secretions of human and canine stomachs as well as the small intestine of dogs, are devoid of enzymes acting upon pectin. On the other hand pectin incubated with a suspension of feces is rapidly decomposed. This suggests that the pectin passes through the esophagus, stomach and small intestine without decomposition but is subsequently decomposed by the enzymes produced by the abounding flora of microorganisms rather than by enzymes excreted by the large intestine.

Obviously the validity of these conclusions depends on the acceptance of certain assumptions. There is some doubt as to whether the observations made on dogs may be used in predicting the fate of pectin in human beings. Nutritional and other conditions may also be of importance. Murer and Crandall ('40) mention that pectin fed to fasting animals is mostly excreted. Excretion of pectic materials is also apparent when the apple diet is used on patients (or animals) suffering from diarrhea. Perhaps in such cases the normal bacterial flora in the large intestine is altered; this results in a failure to decompose the pectin. There is little excretion of enzymes in the large intestine and it appears safe to assume that the pectin reaching the colon is decomposed there by bacterial enzymes.

The matter of carbohydrate absorption from the large intestine is still a controversial subject. The observations of Imhäuser ('32) that pectin orally administered to dogs does not increase the blood sugar make it probable that the galacturonic acid derived from the pectin is fermented rather than absorbed. Imhäuser also states that the decomposition products of pectin were not excreted. Tests conducted on the fermentation of pectin and galacturonic acid by various intestinal microorganisms (Saunders and Kertesz, '40) indicate that most microorganisms capable of hydrolysing pectin also ferment galacturonic acid.

It is not within the scope of this article to discuss the significance of the present findings in regard to the working mechanism of the apple diet. But it may be suggested that if pectin is not decomposed until it reaches the large intestine its therapeutic action may depend on its colloidal properties. The galacturonic acid derived from the pectin upon hydrolysis may be available for detoxication (Manville, Bradway and McMinis, '36) after absorption from the colon. It is also possible, although not very probable, that some galacturonic acid would reach the ileum by regurgitation (Manville, '40), be absorbed and participate in detoxication reactions.

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SUMMARY

Tests made with human subjects and dogs indicated that saliva and gastric juice do not contain enzymes acting upon pectin. Pectin passed through the stomach and part of the small intestine of a dog could be recovered without loss. Trypsin, pepsin and rennet had no effect on pectin in vitro, but pectin incubated with feces was rapidly decomposed.

It appears probable that pectin taken orally is not attacked until it reaches the large intestine where it is completely hydrolysed by bacterial enzymes.

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RELATIONSHIP BETWEEN INSENSIBLE LOSS OF WEIGHT AND HEAT PRODUCTION OF THE RABBIT

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ONE FIGURE

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Benedict and Root ('26) and Newburgh et al. ('37) have shown that with humans there is a relationship between the insensible loss of weight and the heat production. Benedict and Root state that measurement of the insensible loss of weight alone can be used to estimate the heat production. Gasnier and Mayer ('34) studied the insensible loss of weight of rabbits at 20°C. and found that this loss decreased with fasting, was different with different animals, increased as the environmental temperature rose, and was influenced by other conditions. It is the purpose of this paper to determine to what extent the rabbit's insensible loss of weight at a given temperature is related to its heat production, to note factors that may affect this relationship, and to determine whether measurement of the insensible loss can be used to estimate this animal's heat production.

METHODS

Twelve adult rabbits of various sizes were used in these experiments.[1] The metabolism measurements were made at 28° to 29°C., with the apparatus used by Lee ('39), and for 24 hours beforehand the rabbits were kept at 28°C. A rabbit was weighed in a wire basket on a 5-kg. Sauter balance. It was

[1] The author gratefully acknowledges the technical assistance of Mr. George Lee.

then transferred to the metabolism chamber, and when the chamber had been sealed and ventilated for 3 minutes, the measurements of the gaseous exchange began. There were nine consecutive periods of such measurements, each of 20 minutes' duration. The rabbit was then removed from the chamber and weighed again in the wire basket. The measurement of the insensible loss of weight covered about 3½ hours and that of the metabolism 3 hours. The heat production was calculated from the measurements of the respiratory exchange.

At the beginning of the first period of the respiration experiment equilibrium of the gaseous mixture inside the chamber was probably not fully established, but the metabolism measurements were started early in an effort to have the time covered by such correspond as nearly as possible to the time of measurement of insensible loss. The transfer of the rabbit from the weighing basket to the chamber undoubtedly caused an increased metabolism, and it was desired to include this period in the metabolism measurement, as it was included in the measurement of the insensible loss. The heat production in the first period of each metabolism experiment averaged 13% above the average heat production in all the periods of the experiment.

Only measurements obtained when the rabbit was dry at the start and end of the experiment, when no urine or feces were voided, and when there were no visible indications of moisture from condensation, drooling, or any other source, have been included in the results reported in this paper.

As the ventilating air supplied to the chamber was dried by calcium chloride, the relative humidity of the outcoming air at the average chamber temperature was calculated from the hourly insensible loss (corrected for the carbon-dioxide elimination and the oxygen absorption to obtain the grams of water vaporized per hour) and the volume of air ventilating the chamber per hour. The air entered the chamber near the rabbit's head. Hence because of the slow ventilation rate and because calcium chloride cannot completely dry air, the

average humidity of the chamber was nearly that calculated for the outcoming air.

RESULTS

In table 1 are recorded the results of seventy-four experiments in which simultaneous measurements were made of the heat production and the insensible loss of weight of these twelve rabbits. In six instances the rabbits were measured immediately after eating but in all other instances had been 24 hours without food at the time of measurement and were in the post-absorptive state, as indicated by their respiratory quotients (range, 0.71 to 0.78). When the values for insensible loss of weight per hour are plotted with reference to the hourly heat production under post-absorptive conditions (fig. 1), it is apparent that there is a straight-line relationship between these two factors.

Humidity. As the insensible loss is made up in large part of evaporated water and as evaporation of water is affected by differences in humidity, the question arises as to whether this factor influences the relationship under consideration. With eight rabbits the humidity differed by 20% or more in different fasting experiments, but comparison of the results obtained at the extremes of humidity indicates no tendency for the relationship to be influenced by humidity. When the values for humidity were indicated against the plotted points, the distribution of the high and low humidities above and below the average curve showing the general trend of the data was essentially even. In three experiments (indicated by crosses in fig. 1) in which calcium chloride was placed in the bottom of the respiration chamber and the rabbits rested on a wire grate slightly above it, the humidity of the air, at least in parts of the chamber, was extremely low. These showed an appreciably greater insensible loss than other experiments on the same animals with similar levels of heat production but with normal humidity. Between the limits of 40 to 80%, however, differences in humidity did not measurably influence the relation of insensible loss to heat production.

TABLE 1

Insensible loss of weight and heat production of rabbits[1]

RABBIT,[2] AVERAGE WEIGHT, SEX AND DATE OF EXPERIMENT	RELATIVE HUMIDITY	PER HOUR		RABBIT,[2] AVERAGE WEIGHT, SEX AND DATE OF EXPERIMENT	RELATIVE HUMIDITY	PER HOUR	
		Insensible loss	Heat production			Insensible loss	Heat production
1939–40	%	*gm.*	*Cals.*	*1939–40*	%	*gm.*	*Cals.*
A, 2.20 kg. ♀				G, 3.57 kg. ♀			
Nov. 8	65	2.18	5.36	Oct. 11	76	3.49	7.23
Nov. 17	66	2.53	5.16	Nov. 7	76	3.75	7.60
Nov. 22	75	2.62	5.08	Nov. 15	78	3.51	7.62
Nov. 29	71	2.43	5.23	Nov. 23*	85	4.63	7.99
Jan. 3	53	2.02	4.95	Nov. 24	74	3.96	7.23
Jan. 30	53	2.19	5.13	Dec. 1	68	3.74	6.87
Feb. 7	64	2.68	5.50	Dec. 21	63	4.25	7.18
				Jan. 3	63	2.96	6.77
B, 2.23 kg. ♀				Feb. 7	54	4.26	6.97
Feb. 1	47	1.65	4.51				
Feb. 5	47	1.73	4.61	H, 4.13 kg. ♀			
				Nov. 16	85	4.56	9.22
C, 2.31 kg. ♂				Nov. 22	90	4.75	9.20
Oct. 13	75	2.16	4.91	Nov. 29	95	5.18	9.49
Oct. 13	38	2.20	5.26	Dec. 29	92	5.06	9.02
Oct. 25*	61	3.12	6.21	Feb. 2	81	4.10	8.02
Oct. 26	59	1.96	5.43	Feb. 5	84	3.81	7.71
Oct. 26	34	2.13	5.54				
Oct. 31	50	2.12	4.78	I, 4.24 kg. ♀			
Nov. 7	46	1.83	4.57	Nov. 4	59	3.20	8.00
Nov. 15	49	1.70	4.70	Nov. 9	76	3.94	8.41
Nov. 20	43	1.61	4.66	Nov. 9	51	4.13	8.44
Dec. 27*	47	2.04	5.10	Nov. 16	61	3.63	8.60
Dec. 28	43	1.57	4.67	Nov. 22	76	4.00	8.40
Jan. 9	38	1.57	4.44				
				J, 4.31 kg. ♀			
D, 2.40 kg. ♂				Nov. 6	36	1.99	6.02
Nov. 7	54	2.15	5.33	Nov. 10	38	1.90	6.10
Nov. 16	57	2.16	5.57	Nov. 20	39	2.03	6.60
Nov. 22	57	2.01	5.27				
Dec. 1	45	1.50	4.77	K, 4.83 kg. ♂			
Dec. 29	68	1.88	5.90	Nov. 1	58	4.11	7.93
Dec. 29	38	2.03	5.79	Nov. 7	71	3.91	8.41
Feb. 1	49	1.89	5.19	Nov. 10	56	3.08	7.03
				Nov. 21	65	3.38	7.27
E, 3.37 kg. ♂				Dec. 19*	72	5.02	9.19
Nov. 17	66	3.60	6.93	Jan. 16	53	3.56	8.18
Nov. 25	79	4.17	7.94				
Dec. 1	62	3.38	7.09	L, 4.94 kg. ♀			
Jan. 4	67	4.13	7.72	Oct. 16*	71	4.44	10.00
Feb. 1	87	4.53	8.76	Oct. 17	45	3.84	9.10
Feb. 8	66	4.72	7.81	Nov. 9	71	3.66	8.67
				Jan. 17	65	3.87	8.75
F, 3.38 kg. ♀							
Oct. 10	52	2.48	5.98				
Oct. 16*	58	2.80	6.65				
Oct. 17	69	2.30	6.09				
Oct. 17	40	2.54	6.33				
Nov. 6	43	2.13	5.57				
Nov. 14	53	2.30	5.84				
Nov. 20	47	2.46	6.08				

[1] Measured at 28° to 29°C., after fasting 24 hours at 28°C., except in those experiments marked with an asterisk, which were made immediately after food ingestion.

[2] The breed of rabbits A, B, C and D was Dutch, of rabbit E Chinchilla, and of all the others New Zealand White.

Fed versus fasting state. A few rabbits were measured immediately after eating and again 24 hours later, with no additional food intake. In these experiments the heat production was naturally greater on the first than on the second day. When these results were plotted and the values for the 2 days connected by a line, the slopes of these lines were consistently steeper than the average trend of all the data except in one instance, in which case the slope was the same as the average. Hence the relationship between insensible

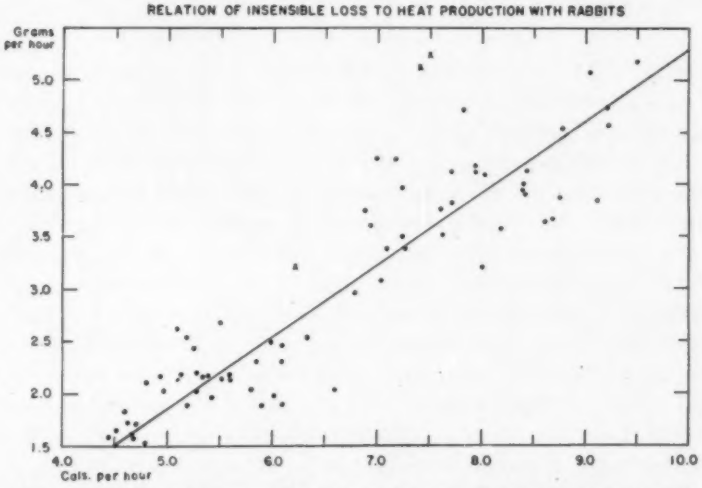

Fig. 1 Relationship between hourly insensible loss of weight and heat production per hour, simultaneously measured, with rabbits fasting 24 hours, at 28° to 29°C. The dots represent the values obtained at relative humidities between 34 and 95% and the crosses, the values obtained in experiments with very low humidity when calcium chloride was placed in the bottom of the respiration chamber.

loss and heat production was slightly different after food ingestion from that after 24 hours of fasting.

Body temperature. There was no consistent effect of differences in the normal level of body temperature, between 38.9° and 41.1°C., upon the relationship. The maximum variation in the body temperatures of the individual rabbit was frequently found when the relationship of the insensible loss of

weight to the heat production remained unchanged. There was also no tendency for the results obtained with rabbits having low or high average body temperature to be definitely below or above the average curve in figure 1. Hence no effect can be attributed to differences in normal body temperature.

Insensible loss as an index of heat production. Between the limits of 1.5 and 5.0 gm. of insensible loss per hour the straight-line relationship between this factor and the heat production of the adult rabbit, when post-absorptive, at 28° to 29°C., may be expressed by the equation

$$H = 1.45L + 2.32$$

in which H equals kg.-calories per hour and L equals grams of insensible loss of weight per hour. By this equation the heat production has been calculated from the measurements of the insensible loss, and the percentage deviations of the measured heat productions from these calculated or predicted heat values have been determined. In two-thirds of the sixty-eight experiments the individual percentage deviations for any given rabbit were within $\pm 5\%$ of its average percentage deviation. The standard deviation of the percentage differences for all the measurements is $\pm 8.9\%$. Measurements of the insensible loss of rabbits under the conditions set forth will serve for approximate calculation of the probable heat production prevailing at the time of such measurements but cannot replace accurate respiratory exchange measurements.

Percentage of heat loss in vaporization of water. The water lost by vaporization has been calculated for these experiments by correcting the insensible loss of weight for the carbon dioxide given off and the oxygen absorbed. From these results the percentages of heat lost through the path of water vapor have been calculated, on the basis that each gram of water vapor represents the loss of 0.576 kg.-calories. The ranges of these percentage losses in the individual experiments with the different rabbits and the average loss of each rabbit are shown in table 2. The average losses vary from 17.7 to 29.7% and average 24.6%. Computing similar values from the data of Mayer and Nichita ('29) obtained in experiments at environmental temperatures of 27° to 29°C., but with

rabbits not fasting, we find losses of 22.8, 24.5 and 27.2%. The two experiments of Marshall, Aydelotte, and Barbour ('31) at 30°C. with rabbits presumably fasting (R.Q. = 0.73 and 0.70) give 24.6 and 25.8%. Our data, therefore, support these fragmentary data. The percentages of heat lost by vaporization of water by other animals measured at thermic neutrality by the Nutrition Laboratory (Benedict, '36; Benedict and Lee, '36, '37 and '38; Ritzman and Benedict, '38) were: albino mice 18%, geese 22%, marmots 20%, cows 26% and an elephant 19%. The average value found by Newburgh et al. for man is 25%.

TABLE 2

Percentage of heat lost by rabbit in vaporization of water[1]

RABBIT	HEAT LOST IN WATER VAPOR		
	Minimum	Maximum	Average
	%	%	%
A	22.9	29.5	25.9
B	21.0	21.5	21.3
C	19.1	25.5	22.1
D	17.6	22.5	20.5
E	26.7	33.7	29 6
F	21.2	24.1	22.6
G	24.5	34.0	29.7
H	27.9	32.2	29.7
I	22.8	28.1	25.9
J	17.4	18.1	17.7
K	24.2	29.1	26.4
L	23.0	24.0	23.5
Average			24.6

[1] Calculated from data in table 1, as explained on page 302.

SUMMARY

Sixty-eight simultaneous measurements of the insensible loss of weight and the heat production of twelve adult rabbits, at 28° to 29°C., after fasting 24 hours at 28°C., showed that there was a relationship between these factors. This relationship was not measurably affected by variations in humidity in the range of 40 to 80%. Shortly after feeding, the relationship changed slightly. Fluctuations of the relationship could not be correlated with changes in normal body temperature.

Between the limits of 1.5 and 5.0 gm. of insensible loss per hour, the relationship was a straight-line function that can be expressed by the equation

$$H = 1.45L + 2.32$$

in which H equals kg.-calories per hour and L, grams of insensible loss per hour. The standard deviation of the percentage differences of the measured heat production from the heat production calculated from the insensible loss by this equation is $\pm 8.9\%$. Measurement of the rabbit's insensible loss of weight under the conditions set forth will give approximate values for the probable heat production prevailing at the time of measurement but will not replace exact measurements of the respiratory exchange. The heat lost by vaporization of water, as shown by indirect calculation, averaged 24.6% of the measured heat production.

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